21, 1996. Applicants enclose a copy of this European Search Report to show the Examiner that both WO 92/11358 and Young *et al.* cited in the search report are also cited on the PTO 1449. EP-A-0 279 582 was cited on the PTO 1449 submitted on December 21, 1992. Therefore, the three publications cited in the European Search Report have been individually cited on PTO 1449s. It is requested that the Examiner cross out reference to the European Search Report for Appln. No. 9204523.5 on the PTO 1449 submitted on June 21, 1996.

Rejection of Claims Under 35 USC §112, First Paragraph

Claims 17, 20, 21 and 24 are rejected under the first paragraph of 35 USC §112, on the basis that the specification does not enable "the production of any protein in the mammary gland for eventual isolation from the cow's milk ... nor are there sufficient teachings in the art for the production of such a transgenic cow." Applicants respectfully traverse the basis for rejection.

The examiner has stated that:

> ... Applicant argues that it is not true that growth to morula/blastocyst stage prior to implantation is necessary. Applicant argues that both skills (direct transfer of microinjected zygotes to the recipient cow and incubation of the microinjected zygote to the morula/blastocyst stage *in vitro* prior to transfer to the recipient cow as disclosed in Krimpenfort *et al.*) were known to the artisan at the time of filing. These arguments are not persuasive.
>
> A declaration may be found non-persuasive if the opinions offered by the expert declarant, such as Dr. Bondioli, are refuted by evidence in the art. In the previous office action, and in this office action, it is maintained that the art does not support declarant Bondioli's opinions. ... Declarant's statements that the issues were economic rather than functions are not persuasive against the backdrop that the only cows argued to produce a heterologous protein in their milk were produced by a method that required the incubation of the microinjected zygote to the morula/blastocyst stage prior to transfer to the mother.

Office action at page 3. Portion in parentheses added.

A. Techniques for producing transgenic cattle were known in the art at the time of filing the present application

First, Applicants note that neither their specification nor claims are limited to producing a transgenic cow by directly transferring the microinjected embryo to a recipient female. Secondly, the claimed invention is enabled by both the specification alone and in combination with techniques that were known in the prior art prior to filing the present application.

As of the September, 1992, the filing date of the present application, methods for the production of transgenic cattle had been reported in the scientific literature and available to persons skilled in the art. It is accepted that the publications directed to producing transgenic cows or sheep did utilize either an *in vivo* or *in vitro* incubation step prior to implanting the embryos into recipient females to carry the fetuses to term. But it must be emphasized that these techniques were known, published and available to skilled persons to use in making transgenic animals, including livestock, such as cattle, sheep, and goats. Therefore, this information, combined with the knowledge of one of ordinary skill in the art, and the present disclosure regarding the constructs containing the 4.2 kb *Sau*3A - *Kpn*1 promoter of the mouse WAP gene, provides an enabling disclosure to produce any protein under the control of this promoter in transgenic cattle.

As a matter of law, the specification need not teach, and preferably omits, what is well known in the art. *See Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). Furthermore, the specification only has to enable a person of skill in the art to practice the claimed invention without undue experimentation. *Id.* Absolute predictability of the outcome is not required. The level of skill in the transgenic art is very high, the methods to practice the invention were known, and considerable experimentation to produce transgenic animals was routine. Applicants recognize, as would others skilled in the art, that

when livestock animals are utilized, the known methods disclosed in these publications could be utilized.

The additional incubation steps after microinjection of the embryos are not so difficult and the results not so unpredictable that a skilled artisan in the transgenic field could not perform these steps without undue experimentation. The additional step of *in vitro* incubation would not require extraordinary skills in embryo manipulations. All that is required is to place the microinjected embryos into a petri dish after microinjection as per the disclosure in any of these publications and incubate them until they reach a predetermined stage of development, which is readily discernible by a skilled person. In regard to *in vivo* incubation of the microinjected embryo, steps of flushing the embryos from the surrogate would be required but these skills would also not require undue experimentation. Failure to specifically delineate these known steps in the present application does not deprive the specification of its enabling character for producing transgenic cattle containing a stably integrated transgene.

For example, each of WO 91/08216 published June 13, 1991; Krimpenfort *et al.*, *Bio/Technology* 9:844-847 (September 1991); Hill *et al.*, *Theriogenology* 37:222 (January 1992); Massey, *J. Reprod. Fert. Suppl.* 41:199 (1990), and Biodioli *et al.*, in *Transgenic Animals* pp 265-273 (1991), all of which are of record in the present application, were published and available to the skilled artisan before the filing date of the present invention. These publications are evidence that prior to the filing date of the present invention, any of the known methods of producing transgenic cattle were available to Applicants to produce transgenic mice, rats, rabbits, pigs, sheep, goats and cows capable of expressing any known protein under the control of the claimed mouse WAP promoter.

Applicants wish to direct the Examiner to the specification on page 15, lines 10-14, where the preferred method of introducing DNA into embryos is microinjection. It is also

stated that these microinjected embryos are then "...allowed to develop into mature transgenic animals." There is no limitation that the microinjected embryos be handled in a particular manner. It is acknowledged that the specific examples disclosing the production of mice and pigs show the direct transfer of microinjected embryos to recipient females; however, the specification **does not limit the handling of microinjected embryos to the direct transfer method.**

Further, Dr. Bondioli's declaration does not characterize the incubation of microinjected embryos as critical to the production of transgenic cattle. But rather he states in paragraph 3 of his declaration that it is his expert opinion that centrifugation of zygotes was critical to producing transgenic cattle as follows:

> In 1987, I participated in a transgenic cattle project as an employee of Granada BioSciences, Inc....The "major breakthrough" which made the production of transgenic cattle possible at that time was the technique of centrifuging zygotes to allow visualization of pronuclei. This method was published by Wall *et al.*, *Biol. Reprod.* 32: 645-651 (1987), and it is described in the Lubon application.

Applicants' examples on pages 28 and 29 of the specification directed to producing a transgenic pig discloses this centrifugation technique.

Dr. Bondioli's review of the specification and his experience as an expert in producing transgenic cattle led him to conclude that:

> [T]he scientific knowledge available as of January of 1991 in combination with the disclosure of the above-captioned application would have enabled a researcher to produce a transgenic cow having a transgene under the control of the "long WAP promoter."

Dr. Bondioli's Rule 132 Declaration at paragraph seven. Applicants submit that the above recited conclusion of Dr. Bondioli took into account what was known prior to the filing of the present invention regarding the incubation of the

microinjected embryos. In fact, Dr. Bondioli states that all of the work from Granada BioSciences, Inc. used the techniques as disclosed in his Exhibits C-E. As the Examiner herself points out, these cited publications used *in vitro* or *in vivo* incubation of microinjected embryos prior to transfer to recipient females. The Examiner is mistaken to read Applicants' invention as excluding the use of these prior art methods. Thus, when Applicants' specification and claims are interpreted properly, it is clear that the art supports rather than undermines the enablement of the claimed invention.

In fact, the integration and expression of transgenes in cattle had been demonstrated and reported in the scientific literature before the date of Applicants' invention. Therefore, a person skilled in the art could make a DNA construct containing the disclosed mouse WAP promoter operably linked to a known DNA sequence encoding a polypeptide for microinjection into a cow embryo using published methods without undue experimentation.

B. <u>The specification enables producing a polypeptide under the control of the claimed WAP promoter in cows</u>

The Examiner only rejects claims that are directed to the expression of "a polypeptide" under the control of the claimed mouse WAP promoter in cows. Applicants assume that the Examiner believes that the introduction of DNA encoding any protein other than protein C into cow embryos is not enabled by the present disclosure.

Applicants point out that several genes encoding diverse heterologous proteins were stably integrated into the DNA of cows as of the filing date of the present application, as evidenced by Krimpenfort *et al.* (lactoferrin), Hill *et al.* (human estrogen receptor and insulin-like growth factor-I) and Massey (human estrogen receptor). Further, documents provided in Applicants' previous response of July 14, 1997 show that the Krimpenfort method resulted in transgenic cows sired by "Herman"

the bull that expressed lactoferrin in their milk. Specifically, the exhibits to the July 14, 1997 response provide a paper trail to show that the method disclosed in the Krimpenfort publication of September 1991 results in a dairy cow that expressed lactoferrin in her milk. Time is needed to produce a cow expressing a polypeptide encoded by the transgene. The evidence provided in the exhibits shows that ultimate expression of the polypeptide in milk was achieved by the methods disclosed in September 1991. No other experimentation was required. Only breeding the animals, screening for transgenic calves, and eventually screening for the production of the polypeptide in the milk were required. Also see the attached abstract from the IBC's Third Annual International Symposium on "Producing the Next Generation of Therapeutics Exploiting Transgenic Technologies" (Exhibit 1).

In another example of the expression of the integrated transgene in cows, Bowen *et al.*, *Biol. Reprod.* 50: 664 (1994), (Exhibit 2), reported that several months after birth, a transgenic calf developed dramatic muscular hypertrophy followed by muscle degeneration. The authors concluded that this phenotype was associated with the expression of the transgene.

Obtaining a stable transgene is the important first step in producing founders and then a herd of cattle that express the transgene in their milk. But once the transgene is integrated, known methods for identifying the transgenic animals and breeding them to obtain offspring that express the transgene does not require undue experimentation. It only requires time and money to obtain a herd of producers of the protein of interest.

C. Transgenic livestock were produced by the direct method of transferring embryos to recipient females after microinjection

Prior to the filing date of the present invention, production of heterologous polypeptides in milk had been

achieved in goats and sheep, species closely related to cattle, using the direct transfer method of microinjected embryos.

Ebert *et al.* (Exhibit 3) enclosed herewith, shows the expression of human tPA in the milk of goats under the control of the murine WAP promoter. The Examiner is directed to page 836, second column, first complete paragraph, where it is stated that the microinjected embryos are "...either immediately transferred to the oviducts of recipient females or cultured in Ham's F12 medium ... for 72 hours and subsequently transferred to the uterus of recipient females. See Table 1 where 2 animals out of 22 offspring were transgenic as a result of the direct transfer method. Pages 837 and 838 of this publication show that the founder female produced by the direct embryo transfer method produced human tPA in its milk.

Wright *et al.* (Exhibit 4) disclose the expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. On page 833, second column, under the section "Experimental Protocol", it is recited that transgenic sheep were generated essentially as described in Simons *et al.* (Exhibit 5). Copies of both of these publications are herewith enclosed. Simons incubates microinjected eggs for at least 30 minutes to assess any damage to the eggs prior to transfer to the recipient females. There is no indication that the embryos were incubated to reach the morula/blastocyst stage. Wright does not appear to modify this procedure. Thus, Wright provides evidence of the production of human alpha-1-antitrypsin in the milk of transgenic sheep utilizing the direct transfer method.

In view of these publications, there would be a reasonable expectation that a transgenic protein could be produced in the milk of cattle using the direct transfer method of embryo manipulation.

Applicants believe that they have provided evidence and arguments to support their position that their specification enables the skilled artisan to produce a transgenic cow that expresses a polypeptide under the control of the claimed long

WAP promoter using either of the disclosed methods of handling microinjected embryos. Given Applicants' experience with transgenic mice and pigs, one skilled in the art with the specification and techniques known in the art would be able to carry out the procedures described in the specification to produce a transgenic cow capable of expressing a polypeptide under the control of the claimed long WAP promoter with a high level of confidence of success.

In light of the remarks above, Applicants respectfully request the examiner to withdraw the rejection of the claims under the first paragraph of 35 USC §112.

CONCLUSION

Applicants request reconsideration of the claims on their merits and respectfully solicit early notification of an allowance. If Examiner Crouch should have any questions or believes a telephone discussion would expedite prosecution, she is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,

March 24, 1998
Date

Jayme A. Huleatt
Reg. No. 34,485

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INTERNATIONAL BUSINESS COMMUNICATIONS

International Business Communications

Exhibit 1

IBC's Third Annual International Symposium on

PRODUCING THE NEXT GENERATION OF

THERAPEUTICS

EXPLOITING TRANSGENIC TECHNOLOGIES

February 5-6, 1997, PGA National Resort & Spa, West Palm Beach, FL

1332

Name: _____

Company: _____

ibc Infoline

225 Turnpike Road, Southborough, MA 01772-1749, USA Tel: (508) 481-6400

Novel Data on the Production of Recombinant Human Lactoferrin in Transgenic Cattle - From Technical Challenge to Commercial Reality.

Summary of Presentation by Jan H. Nuijens, MD, PhD, Pharming BV, Niels Bohrweg 11-13, 2333 CA Leiden, The Netherlands.

Human lactoferrin (hLF) is an highly cationic, iron-binding glycoprotein (Mr 77,000) of the transferrin family. Human LF was first isolated from milk, but has also been found in many other external secretions such as tears and saliva, as well as in the specific granules of neutrophils. Extensive *in vitro* and some *in vivo* evidence indicates that hLF participates in host defense against infection, iron metabolism and modulation of inflammatory and immune responses, most notably at mucosal surfaces such as those of the gastroinrestinal tract. Most anti-infective and anti-inflammatory activities of hLF are mediated through sequestration of iron and/or interaction with microbial cell wall components and specific receptors on intestinal epithelial cells and lymphocytes, through its highly positively charged N-terminus.

We have chosen to produce recombinant hLF in the milk of transgenic cows. Mammary gland specific expression vectors based on regulatory elements from the bovine αS1 casein gene and either hLF cDNA (Krimpenfort et al, 1991, Bio/Technology 9: 844-847) or the genomic hLF sequences were introduced into the bovine germline by pronuclear injection of one-cell stage embryos. The latter genomic hLF constructs were selected after a process of construct optimization and evaluation in transgenic mice, which expressed hLF in a mammary gland-specific and lactation-restricted fashion in milk (Platenburg et al, 1994, Transgenic Res. 3: 99-108). Human LF was found at relatively low levels in the milk of hLF cDNA transgenic cows, but at levels in the g/L range in the milk of the first lactating genomic hLF founder cow. Based on the correlation of transgene copy number and hLF expression level in transgenic mice, we expect that the hLF expression levels from the other founder animals harbouring a higher copy number will even be higher. The expression of hLF did not affect milk quantity or composition, nor was associated with changes in health or welfare of the lactating cows. Comparison of recombinant hLF with hLF from human milk revealed the proteins to be virtually identical by immunological (double antibody assays; hyperimmunization experiments), functional (iron-binding and -release; binding to LPS, heparin and other physiologically relevant ligands) and structural criteria (analytical chromatography; protein sequencing; spectroscopy; utilization of glycosylation sites).

Based on these observations, we anticipate that recombinant hLF will exert similar if not identical antimicrobial and anti-inflammatory actions *in vivo*. We are currently generating herds for the large-scale production of hLF and focussing on the pharmaceutical, preclinical and clinical development of the protein to evaluate its therapeutic and prophylactic potential in several human pathological conditions.

Feb. 1997

BIOLOGY OF REPRODUCTION 50, 664–668 (1994)

Transgenic Cattle Resulting from Biopsied Embryos: Expression of c-ski in a Transgenic Calf

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ABSTRACT

Producing transgenic cattle by microinjection of DNA into pronuclei has been inefficient and costly, in large part because of the cost of maintaining numerous nontransgenic pregnancies to term. We designed a system for early identification of transgenic embryos in which biopsies of embryos were assayed by polymerase chain reaction for presence of the transgene before embryo transfer. A total of 2555 embryos were microinjected with one of two DNA constructs. Of the 533 embryos biopsied, 112 were judged to be potentially transgenic and were transferred nonsurgically to recipients, resulting in production of 29 putative transgenic fetuses. One fetus and one calf (7% of offspring) were subsequently shown to be definitively transgenic. The calf was transgenic for a chicken c-ski cDNA, and several months after birth developed dramatic muscular hypertrophy followed by muscle degeneration. This phenotype was associated with expression of high levels of mRNA from the transgene.

INTRODUCTION

The success of modern animal agriculture is largely a result of many centuries of selection for desirable genotypes. With the advent of transgenic technology, it became appealing to consider accelerating the pace of such genetic progress, including modifying the genome of animals in ways that probably cannot be done by classical means. Cattle represent a very substantial fraction of world agricultural output and are therefore an attractive target for genetic manipulation. However, progress in producing transgenic cattle is constrained by a number of factors, including long generation interval, low fecundity, and the expense of such work. Nevertheless, a small number of transgenic cattle have been produced in recent years [1–3]. To date, none of these transgenic cattle has been reported to express RNA or protein from the transgene.

A large part of the expense in conducting transgenic research with livestock is in maintaining to term the recipient animals that harbor nontransgenic fetuses. This is a particularly serious problem with cattle because efficiency, in terms of the fraction of pregnancies that are transgenic, has been very low, and the cost of maintenance is high. Considerable savings could be realized if embryos could be reliably screened for transgenic status before transfer. Here we report production of a transgenic calf and a transgenic fetus derived from embryos that were biopsied prior to embryo transfer to determine transgenic status.

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MATERIALS AND METHODS

Embryo Manipulation

Embryos for microinjection were obtained by either surgical recovery from superovulated cows or by in vitro maturation and in vitro fertilization of oocytes aspirated from ovarian follicles of slaughtered cows. In the former case, cows were superovulated according to a protocol similar to that previously described [4], consisting of intramuscular injections at half-day intervals of 6, 6, 4, 4, 2, 2, 2, and 2 mg of FSH (FSH-P; Schering-Plough, Kenilworth, NJ) beginning near midcycle. Prostaglandin $F_{2\alpha}$ was administered to induce luteolysis, and 100 μg of GnRH was administered intramuscularly at the onset of estrus. Animals were artificially inseminated one-half day and one day after the onset of estrus. Donor cows were anesthetized 48–54 h after the onset of estrus and subjected to midline laparotomy, and embryos were recovered by retrograde flushing of the oviducts with Dulbecco's PBS supplemented with 0.3 mM pyruvate and 0.2% BSA. Embryos derived from in vitro fertilization were generated essentially as described [5] and were microinjected 18–26 h after mixing of oocytes and sperm.

Microinjection was conducted similarly to what has previously been described for pig and sheep embryos [6]. One-cell embryos were centrifuged for 3 min at 12 500 × *g* to polarize cytoplasmic lipid droplets, and then microinjected into one pronucleus with a solution of the DNA fragment (2 ng/μl in 10 mM Tris [pH 7.4], 0.2 mM EDTA). Two-cell embryos were treated similarly and injected into one or both nuclei. The majority of embryos were then processed through the following system. After microinjection, embryos were placed in 50-μl drops of oviduct epithelial cell-conditioned medium [8] under paraffin oil and cultured overnight. The next day, cleaved embryos were transferred to the ligated oviducts of estrous rabbits. Embryo

were recovered from rabbits 6–7 days later (equivalent to 9–10 days from the onset of donor estrus) and evaluated for stage of development. Morulae and blastocysts were microsurgically biopsied by techniques similar to those previously described for producing identical twin cattle [9]. Briefly, embryos were placed in a 10-cm plastic Petri dish containing protein-free PBS, resulting in sticking of zonae pellucidae to the plastic surface. With the aid of a micromanipulator, a small fragment of razor blade attached to a glass pipette was used to cut a biopsy from the side of the embryo, avoiding the inner cell mass. Typically, the biopsy was estimated visually to consist of 10–30 of the embryo's trophoblast cells. While the biopsied embryos remained in culture, the biopsies were analyzed for the presence of the transgene by polymerase chain reaction (PCR). Embryos classified as strong or weak by PCR were transferred nonsurgically to the uterus of recipient cows that generally were one day behind the donor cow in estrous cycle synchrony. In a majority of cases, single embryos were transferred, but often one or two poor-quality embryos were transferred with a good embryo to the same recipient. Pregnancy was diagnosed by ultrasonography 30–40 days after transfer. Most of the recipients that became pregnant after transfer of putative transgenic embryos were subjected to allantocentesis 70–90 days after transfer. For this procedure, a flank laparotomy was performed under local anesthesia, and 20–30 ml of allantoic fluid was aspirated through a 20-gauge needle attached to a piece of polyethylene tubing; the first 5–10 ml of fluid was discarded to minimize contamination with maternal cells. Fetal cells were collected from the fluid by centrifugation, counted, and assayed by PCR. Fetuses from other recipients were either recovered surgically between 90 and 130 days of gestation or allowed to go to term.

Transgenes

Embryos were injected with one of two DNA sequences. The first was a construct designed to express the bovine leukemia virus (BLV) envelope protein under control of an immunoglobulin regulatory element (IgSVBLVenv), and was constructed with use of standard techniques by ligating sequences encoding the BLV gp51 gene into an expression vector consisting of the SV40 early promoter and mouse immunoglobulin heavy chain enhancer (Schnieke, unpublished). Expression of gp51 from this construct in cultured cells was found to be very poor after its use for microinjection into bovine embryos was initiated. The second construct, MSVski/Δ29, consisted of a truncated form of chicken c-ski under transcriptional control of a murine sarcoma virus regulatory region [10, 11]; this construct was provided by Dr. Pramod Sutrave and Stephen Hughes (NCI, Frederick, MD). For microinjection, both constructs were released from plasmid sequences, isolated from agarose gels, and purified by adsorption and elution from glass particles.

PCR and DNA Hybridizations

Embryo biopsies (approximately 10–30 cells) and cells collected by allantocentesis (approximately 500 cells) were placed in 0.5-ml polypropylene tubes in less than 5 μl of PBS. PCR was initiated by adding a mixture of buffer and primers and heating the samples to 95°C for 7 min in a thermocycler (Perkin-Elmer-Cetus, Irvine, CA). The samples were then cooled to 80°C and held at that temperature while nucleotide triphosphates and *Taq* polymerase was added. Samples were then subjected to 35 cycles of amplification (94°C/1 min, 55°C/1.5 min, 72°C/2.5 min) with a final 10-min extension at 72°C. Final concentrations of reactants in a 100-μl volume were as follows: single-strength buffer, 1.5 mM MgCl, 200 μM for each nucleotide, 200 nM for each primer, and 2.5 U *Taq* polymerase. Positive (plasmid) and negative (no DNA) control samples were included in each assay. The primers used for detection of MSVski were 5′-AAGGAATTCTCTAGCACGATTGAG (forward) and 5′ CTCAGTAGAAGCTGGAGATTG (reverse). At completion of amplification, 25–30 μl of each product was electrophoresed in 2% agarose containing ethidium bromide, and fluorescent bands of the appropriate size were scored qualitatively as clearly positive or questionable (faint).

Extraction of genomic DNA and Southern blot analyses were conducted according to standard procedures [12]. Ten-microgram samples of DNA were restricted overnight, electrophoresed in 0.8% agarose, and transferred to nylon membranes (Gene Screen Plus, Dupont, Wilmington, DE) by capillary blotting. These samples were hybridized overnight to chicken c-ski cDNA probes labeled with ^{32}P by the random priming method. After a final wash in single-strength saline sodium citrate/0.1% SDS at 55°C, blots were subjected to autoradiography for 1–3 days.

RESULTS

Production and Screening of Transgenic Embryos

A total of 1573 surgically collected and 982 in vitro-fertilized embryos were microinjected with DNA. Of these, 875 embryos were injected with IgSVBLVenv, a construct designed to express the bovine leukemia virus envelope glycoprotein, and 1680 were injected with MSVski, a vector for expression of a truncated form of chicken c-ski. Of 2046 embryos transferred to rabbits, 1535 (75%) were recovered, and 533 of those had developed into blastocysts of quality sufficient to biopsy (approximately 32% and 15% of the transferred embryos derived from oocytes fertilized in vivo and in vitro, respectively). Transfer of 112 PCR-positive or PCR-questionable embryos into 88 recipients (66, 20, and 2 transfers of one, two or three embryos, respectively) resulted in establishment of 27 pregnancies with 29 fetuses. Twelve of these fetuses were derived from embryos injected with IgSVBLVenv and 17 from embryos injected with MSVski DNA. To investigate the possibility of false negative diagnoses, 84

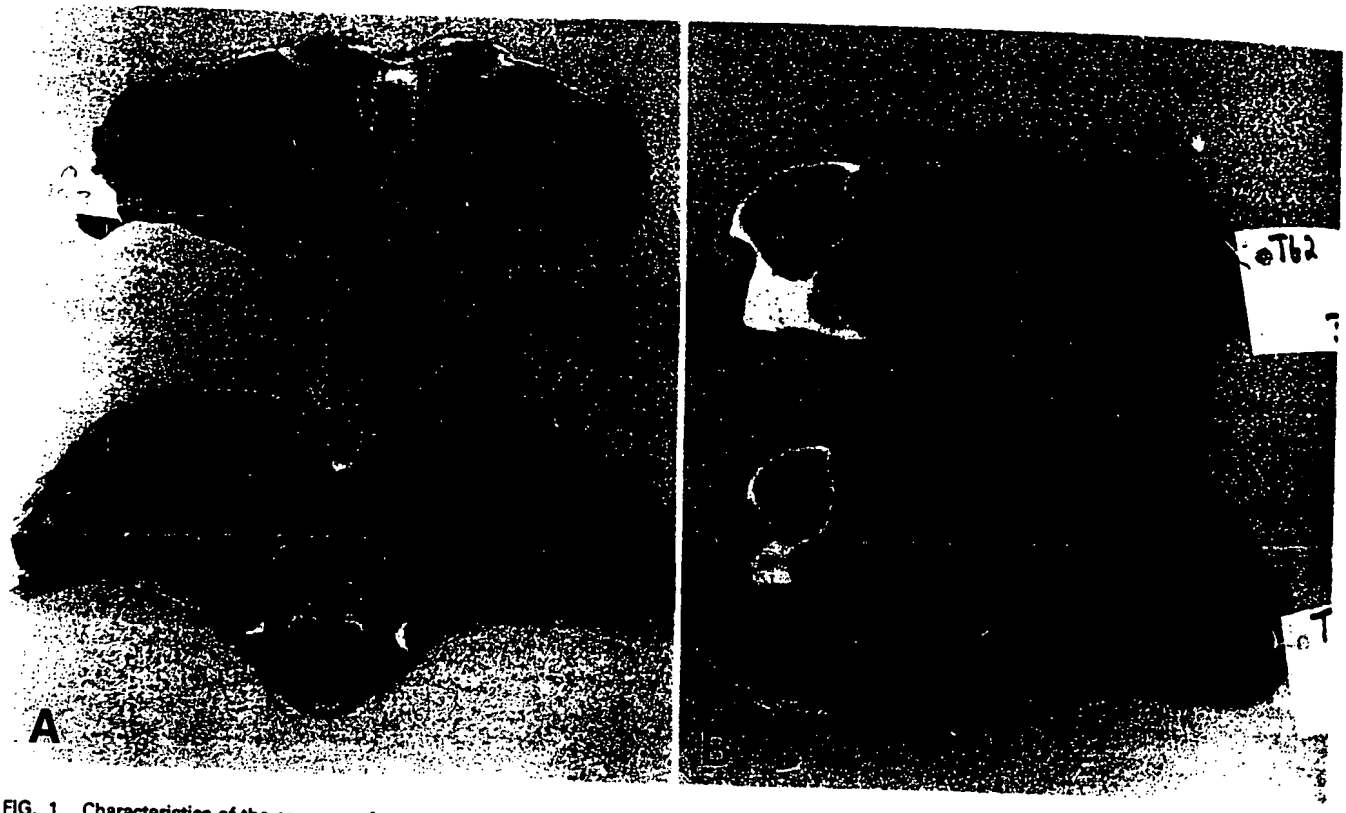

FIG. 1. Characteristics of the carcasses from ski-transgenic calf (bottom of pairs) and his nontransgenic twin sister (top of pairs; sections are at slightly different planes in the carcass). A) Section between 12th and 13th ribs; major muscle is longissimus dorsi. B) Section through shoulder at level of scapulo humeral joint.

biopsied embryos that had been microinjected with MSVski DNA were subjected to PCR analysis after their biopsy was classified as negative. Two of these embryos were classified as putative transgenics on the repeat assay.

Classifying blastocysts as transgenic or not by PCR was of concern because a positive result might only reflect detection of a small amount of injected DNA that had persisted in the embryo but was not integrated. We therefore chose to evaluate each of the putative transgenic pregnancies again at 60–80 days of gestation. Each of the 12 fetuses derived from microinjection of IgSVBLVenv DNA was recovered surgically, and DNA extracted from multiple tissues (liver, skin, and cotyledon) was evaluated by Southern hybridization. One of these fetuses was shown to be transgenic, with the same pattern of hybridizing restriction fragments in all three tissues. The transgene was not detected in the remaining 11 fetuses of this group.

To assess transgenic status in the fetuses from embryos injected with MSVski, fetal cells collected by allantocentesis were again analyzed by PCR. Cells from one of the fetuses contained DNA that was specifically amplified using c-ski primers. This pregnancy consisted of male and female twins. All 17 MSVski pregnancies were allowed to proceed to term. At birth, a skin or tail biopsy was obtained from each of the calves, and DNA extracted from that sample was examined by PCR with c-ski primers. None of the 15 calves diagnosed as nontransgenic at 60–80 days of gestation was transgenic at birth. However, a specific PCR amplification product was obtained with DNA of both twin calves from the pregnancy identified as transgenic by allantocentesis. Southern hybridization with radiolabeled c-ski cDNA and DNA extracted from skin, and at a later age from liver, was used to show that the male calf was transgenic and that the transgene was intact. The female calf showed a small vulva and vagina, and at necropsy, marked uterine and ovarian hypoplasia, characteristics typical of a freemartin. A very weak hybridization signal was obtained with DNA extracted from her skin and a relatively strong signal from liver DNA, undoubtedly due to the hematopoietic chimerism that usually occurs in twin bovine pregnancies; this calf was not considered to be truly transgenic.

Phenotypic Changes Associated with Expression of c-ski

The c-ski transgenic bull calf was normal at birth except for the presence of mild micro-ophthalmia, a defect that is relatively common in cattle and was most likely not associated with his being transgenic. This calf remained phenotypically normal during the first 8 wk of life. However, over the following 2 wk, indications of muscular hypertrophy became evident, particularly affecting the loins and rear quarters. At approximately 10 wk of age, the bull began to manifest sporadic periods of weakness that, over a 2 wk pe

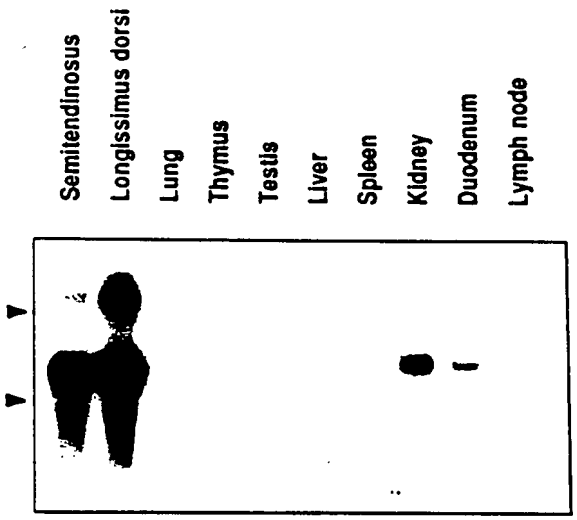

FIG. 2. Northern blot analysis of RNA extracted from tissues collected from ski-transgenic calf. Twenty micrograms of total RNA was loaded in each lane, and blots were hybridized to radiolabeled chicken c-ski cDNA. Arrows mark position of ribosomal RNA bands.

riod, progressed to an inability to stand without assistance. erum concentrations of creatine phosphokinase during this eriod were significantly elevated (ranging from 808–897 /L in comparison to 118–171 IU/L in sera from his non-ansgenic twin), suggestive of skeletal muscle degenera-ion or damage. Throughout this period, the bull remained lert, manifested no signs of pain, and retained a normal ppetite. At 15 wk of age, it became obvious that the muscle eakness was not a transient phenomenon, and humane nsiderations led us to euthanize this animal and, for con-ol purposes, his twin.

Gross evaluation of the carcass from the transgenic bull vealed substantial muscular hypertrophy, which was sym-etrical and involved most of the major muscles associated ith the axial and appendicular skeleton (Fig. 1). The cross-ctional area of the longissimus dorsi muscle between the th and 13th ribs was approximately 40% greater in the nsgenic calf than in his nontransgenic twin (50 cm^2 vs. 36).

Histopathologic examination of major muscles from the nsgenic calf revealed almost all fibers to appear swollen, h a distinctly rounded rather than polygonal outline. A small number of individual fibers were clearly degenerative, with an accompanying mild accumulation of mononuclear cells at their periphery. Many other fibers appeared to be in earlier stages of degeneration, often showing a more acidophilic staining and distinct separation of myofibrils.

Expression of the transgene was assessed by Northern analysis using total RNA (Fig. 2). Messenger RNA for c-ski was present in high concentrations in all axial and appendicular skeletal muscles tested (semimembranosus, semitendinosus, longissimus dorsi, psoas major, triceps, and gluteus medius), as well as in diaphragm. Lesser, but still substantial levels were found in kidney and left ventricle, whereas small quantities were present in small intestine, liver, and spleen. Ski mRNA was not detected in cerebrum, lung, thymus, testis, or lymph node from the ski-transgenic calf, nor in major skeletal muscles (semimembranosus, semitendinosus, and longissimus dorsi) of the nontransgenic twin calf. Transgenic c-ski mRNA was predominantly of the size expected (approximately 2.9 kb), although a larger hybridizing species of unknown origin (approximately 6.5 kb) was observed in muscle samples containing high levels of c-ski mRNA.

DISCUSSION

The study reported here suggests that it is feasible to screen embryos for presence of a transgene prior to transfer into recipients. We generated two transgenic animals from a total of 2555 embryos injected with DNA, an efficiency similar to that reported by other groups using bovine embryos [2, 3]. However, viewed from a different perspective, these two transgenic cattle were derived from 29 fetuses that were obtained from embryos biopsied as blastocysts to assess transgenic status. If the biopsy and PCR step had not been included, we probably would have transferred all 533 good-quality embryos recovered from rabbits to recipient cows (Table 1). Introduction of the embryo biopsy step into the protocol thus did not increase overall efficiency, but it significantly reduced the expense of performing this work by eliminating much of the cost associated with maintaining nontransgenic pregnancies to term.

The PCR technique used for analyzing biopsies is extremely sensitive, but incapable of differentiating integrated DNA from residual input DNA. Short-term persistence in the embryo of small quantities of the microinjected DNA was

TABLE 1. Efficiencies of key steps in producing transgenic calves.

	N	Percent of original embryos	Percent of previous step
Embryos microinjected with DNA	2555	100	100
Embryos transferred to rabbits	2046	80	80
Embryos recovered from rabbits	1535	60	75
Embryos biopsied and subjected to PCR	533	21	35
Embryos transferred to recipient cows	112	4.4	21
Putative transgenic pregnancies	29	1.1	26
Trangenics produced	2	0.1	7

probably responsible for our finding that only 2 of the 29 embryos (7%) that were PCR-positive at biopsy and developed into fetuses were truly transgenic when examined later in gestation. This supposition was supported by PCR on bovine embryos at increasing times after microinjection: 5 of 6, 5 of 6, 5 of 8, 4 of 8, and 2 of 8 embryos were PCR-positive when assayed 1, 2, 3, 5, and 7 days, respectively, after microinjection. Additional experimental support for the problem of plasmid persistence has recently been provided from other laboratories [13, 14]. Despite the problem of false positives, the system of assessing transgenic status in embryos before transfer to recipients reduced the number of recipients needed by 79 percent, as 421 of the 533 good-quality embryos recovered from rabbits were not transferred.

Another important question related to diagnosing transgenic status by embryo biopsy is how frequently the procedure fails to detect truly transgenic embryos, which are then discarded. A substantial percentage of transgenic mice have been found to be mosaics [15]. Integration of a transgene selectively or predominantly into cells that form either inner cell mass or trophoblast could result in a biopsy of trophoblast being falsely classified as negative or positive. We detected the transgene by PCR in 2 embryos from a sample of 84 in which the transgene was not detected in the original biopsy. This could have resulted either from a false negative assay from the biopsy or from transgene mosaicism. Regardless of cause, it is probable that application of the protocol described here will result in discarding an occasional embryo that is truly transgenic. Additionally, there are several ways in which this protocol could be improved, albeit at the cost of increased complexity or time. For instance, a second set of primers could be included as an internal control to co-amplify a segment of bovine genomic DNA. Similarly, Y chromosome-specific primers could be included to sex the embryo, which should allow routine transfer of more than one embryo without the risk of generating freemartins.

The transgenic calf obtained carried a truncated cDNA for the chicken c-ski gene, under regulatory control of murine sarcoma virus promoter and enhancer sequences. This was the same construct used by others to generate transgenic mice [10] and pigs [16]. As might be expected, development of muscular hypertrophy in mice transgenic for chicken c-ski varied with level of expression. The mice that developed muscular hypertrophy showed high levels of c-ski RNA in muscle, whereas those that were transgenic but without the phenotype had low levels of expression. A similar relationship was observed in the c-ski-transgenic pigs. It thus appears that a relatively high threshold of ski gene expression is necessary for development of a hypertrophic phenotype. The ski-transgenic calf reported here supports these observations in that widespread muscle hypertrophy was found in association with high levels of expression of the c-ski transgene. In this calf, as well as in some of the ski-transgenic pigs, high levels of ski gene expression we also associated with muscle dysfunction and degeneratic The pathogenesis of this phenomenon is unclear, partic larly in view of the current lack of understanding of h ski fits into normal patterns of muscle development a interacts with other transcription factors [17–19]. It is like that a window of expression exists in which muscular h pertrophy will develop without impaired function. Belo this window, muscular hypertrophy will not be observe and above it, pathologic changes will ensue. The width this window has significant implications for utility of th gene in transgenic food animals.

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REFERENCES

1. Roschlau K, Rommel P, Andreewa L, Zackel M, Roschlau D, Zackel B, Schwenn M, Huhn R, Gazarjan KG. Gene transfer experiments in cattle. J Reprod Fertil Suppl 1989; 38:153–160.
2. Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van den Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R, de Boer H. Generation of transgenic dairy cattle using "in vitro" embryo production. Biotechnology 1991; 9:844–847.
3. Hill KG, Curry J, DeMayo FJ, Diller KJ, Slapak JR, Bondioli KR. Production of transgenic cattle by pronuclear injection. Theriogenology 1992; 37:222.
4. Bowen RA, Elsden RP, Seidel GE Jr. Embryo transfer for cows with reproductive problems. J Am Vet Med Assoc 1978; 172:1303–1307.
5. Lu KH, Gordon I, Gallagher M, McGovern H. Pregnancy established in cattle by transfer of embryos derived from in vitro fertilization of oocytes matured in vitro Vet Rec 1987; 121:259–260.
6. Hammer RE, Pursel VG, Rexroad CE, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 1985; 315:680–683.
7. Simmons JP, Wilmut I, Clark AJ, Archibald AL, Bishop JO, Lathe R. Gene transfer into sheep. Biotechnology 1988; 6:179–183.
8. Eyestone W, Jones J, First N. Some factors affecting the efficacy of conditioned medium for the culture of early bovine embryos. J Reprod Fertil 1991; 92:59–67.
9. Williams TJ, Elsden RP, Seidel GE Jr. Pregnancy rates with bisected embryos. Theriogenology 1984; 22:521–531.
10. Sutrave P, Kelly AM, Hughes SH. Ski can cause selective growth of skeletal muscle in transgenic mice. Genes & Dev 1990; 4:1462–1472.
11. Sutrave P, Hughes SH. Isolation and characterization of three distinct cDNAs for the chicken c-ski gene. Mol Cell Biol 1989; 9:4046–4051.
12. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning, A Laboratory Manual. Second Edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
13. Ninomiya T, Hoshi M, Mizuno A, Nagao M, Yuki A. Selection of mouse preimplantation embryos carrying exogenous DNA by polymerase chain reaction. Mol Reprod Dev 1989; 1:242–248.
14. Burdon TG, Wall RJ. Fate of microinjected genes in preimplantation mouse embryos. Mol Reprod Dev 1992; 33:436–442.
15. Wilkie TM, Brinster RL, Palmiter RD. Germline and somatic mosaicism in transgenic mice. Dev Biol 1986; 118:9–18.
16. Pursel VG, Sutrave P, Wall RJ, Kelly AM, Hughes SH. Transfer of c-ski gene to enhance muscle development. Theriogenology 1992; 37:278.
17. Colmenares C, Stavnezer E. The ski oncogene induces muscle differentiation in avian embryo cells. Cell 1989; 59:293–303.
18. Nagase T, Mizuguchi G, Nomura N, Ishizaki R, Ueno Y, Ishii S. Repression of a protein co-factor for the DNA-binding function of the human ski proto-oncogene product. Nucleic Acids Res 1990; 18:337–343.
19. Sutrave P, Hughes SH. The ski oncogene. Oncogene 1991; 6:353–358.

TRANSGENIC PRODUCTION OF A VARIANT OF HUMAN TISSUE-TYPE PLASMINOGEN ACTIVATOR IN GOAT MILK: GENERATION OF TRANSGENIC GOATS AND ANALYSIS OF EXPRESSION

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We report the first successful production of transgenic goats that express a heterologous protein in their milk. The production of a glycosylation variant of human tPA (LAtPA - longer acting tissue plasminogen activator) from an expression vector containing the murine whey acid promoter (WAP) operatively linked to the cDNA of a modified version of human tPA was examined in transgenic dairy goats. Two transgenic goats were identified from 29 animals born. The first animal, a female, was mated and allowed to carry the pregnancy to term. Milk was obtained upon parturition and was shown to contain enzymatically active LAtPA at a concentration of 3 μg/ml.

Several types of human proteins have been expressed in lactating mammary glands of transgenic mice and the proteins secreted into milk (reviewed in refs. 1,2). The scale-up of mouse model systems to livestock species may ultimately provide an alternative production system to the commonly used mammalian tissue culture production processes. A transgenic mammary gland production system would have the potential advantages of not requiring an intensive capital expenditure in setting up a manufacturing facility, and of providing a highly cost efficient system due to high expression levels and low expendable production costs.

Most of the work to date has been done in the mouse model system. Regulatory sequences of the whey acid protein (WAP), β- and α-lactoglobin (BLG) and β-casein genes have been used to target expression of numerous genes to the lactating mammary gland, including tissue plasminogen activator (tPA)[3,4], human anti-hemophilic factor IX[5], soluble CD4[6], human interleukin-2[7], and human α_1-antitrypsin[8]. A vector system based on the αS1-casein gene has also been tested in a mouse model where human urokinase was produced efficiently in milk at levels 10 times greater than those achieved in cellular expression systems[9].

Whereas model systems have demonstrated the feasibility of targeting gene expression to the lactating mammary gland and secretion of heterologous proteins into milk, efficient generation of transgenic livestock and production of foreign proteins in their milk have proven more difficult to achieve. Early work aimed at the generation of transgenic farm animals led to low frequencies of integration, low number of animals that expressed the recombinant proteins, reproductive problems, and resultant physiological problems[2]. By targeting protein synthesis to an exocrine organ in which expressed proteins would be expected to be sequestered away from the circulation and removed from the animal, we and others hope to bypass some of these potential problems in the generation of transgenic livestock. In fact, transgenic sheep, which produce factor IX in their milk, have been generated and apparently exhibit no physiological or reproductive problems[5], although expression levels in these animals were low. More recently, however, high expression of the murine whey acidic protein in transgenic swine and mice may have had adverse effects on the physiology of the mammary gland[10].

We have aimed to produce a commercial prototype for the large-scale manufacture of high market-volume proteins in the transgenic mammary gland system using the dairy goat as a production animal. The goat was chosen for several reasons: (1) Dairy goats produce large volumes of milk, on average 4 liters per day; (2) goats have gestation and development periods of moderate length (5 and 8 months respectively); and (3) goat milk has been extensively characterized at the biochemical level[11]. In this paper, we describe the first successful generation of transgenic goats at frequencies that approach those in the rodent systems. More importantly, a transgenic goat was generated that produced an enzymatically active form of tPA throughout a normal lactation period. These experiments further support the concept of targeting expression of transgenes that encode pharmaceutical proteins to the mammary gland of dairy livestock.

RESULTS AND DISCUSSION

Generation of transgenic goats. The expression vector WAP-tPA was generated previously by fusing a 2·6 kb EcoRI-KpnI fragment upstream of the murine whey acid protein gene to a cDNA encoding wild type human tPA[5]. This vector led to expression of tPA in milk of transgenic mice at levels as high as 250 μg/ml (data not shown). A structural tPA variant was constructed (designated LAtPA) in which an asparagine to glutamine point mutation was introduced into the cDNA to produce a recombinant protein devoid of glycosylation at residue Asn 117. This longer acting tPA variant had an increased systemic half-life in a rabbit model[12]. A DNA fragment containing this point mutation in the tPA cDNA was substituted for the equivalent fragment in WAP-tPA to generate the vector used in this study, WAP-LAtPA (Fig. 1).

Goat embryos were flushed surgically from the oviducts of superovulated dairy goats as described in the Experimental Protocol. The superovulation protocol had been

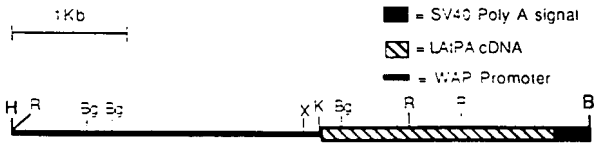

FIGURE 1 A schematic representation of the WAP-LAtPA expression vector and its restriction enzyme sites used for the production of transgenic goats. H = HindIII, R = EcoRI, Bg = BglII, X = XbaI, B = BamHI, K = KpnI.

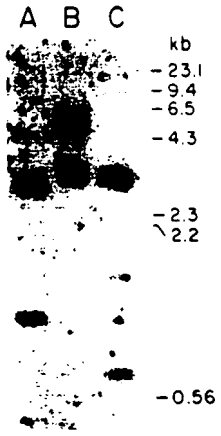

FIGURE 2 Southern blot hybridization of transgenic goat DNA. DNA isolated from the blood of goat #1 was digested, fractionated on an agarose gel, and hybridized to a probe from the whey acid protein gene as described in the Experimental Protocol. Hybridization of this probe to DNA from non-transgenic animals gave no signal (data not shown). Lanes A: BglII; B: XbaI; C: EcoRI.

previously optimized to result in the highest yield and proportion of one-cell embryos[13]. In the course of this study, a total of 372 embryos or ova were collected from 63 donor animals. Of the embryos collected, 252 (68%) were zygotes, 24 (7%) were 2-cells, 5 (1%) were 4-cells and 91 (25%) were unfertilized as determined by the absence of pronuclei. Twenty-eight percent of the injected fertilized embryos were considered poor injections due to the non-optimal positioning of the injected pronuclei, i.e., the pronuclei were at the periphery of the cell making the nuclear injection very difficult. Approximately 8% of the fertilized embryos collected had to be centrifuged for 30 seconds at 13,000 × g to visualize the pronuclei under Normarski optics. Due to the limited number of recipients and donors available on any given experimental day, microinjected 1-cell, centrifuged 1-cell, and 2-cell goat embryos were typically mixed prior to transfer to the recipient females. This prevented us from confirming the viability of the centrifuged eggs or the potential production of transgenic goats by microinjection of 2-cell embryos. However, subsequent experiments with other fusion genes have confirmed that microinjected centrifuged 1-cell goat embryos are viable and can produce viable offspring. In addition, we have also produced a transgenic goat (potentially a mosaic) that was the result of injection of a 2-cell goat embryo (data not shown).

Embryos were injected with a 4.9 kb HindIII-BamHI fragment of WAP-LAtPA purified free of procaryotic DNA (Fig. 1) at a concentration of 1 μg/ml in 10 mM Tris, pH 7.5, 0.1 mM EDTA, and either immediately transferred to the oviducts of recipient females or cultured in Ham's F12 medium containing 10% fetal calf serum in an atmosphere of 5% CO_2 in air at 37°C for 72 hours and subsequently transferred to the uterus of recipient females. Typically the cultured goat embryos were blocked at the 8-16 cell stage in this culture system, but remained viable and could produce live offspring (Table 1).

Pregnancies were confirmed by the inability of recipient animals to return to natural estrus and by ultrasonic examination on days 45 and 55 of pregnancy. Ultrasound on days 45 and 55 of pregnancy have resulted in a 100% early pregnancy confirmation over the first three years of this project. A complete summary of the recipient data is shown in Table 1. Twenty-nine animals were born from 203 embryos transferred, representing 14% of injected embryos surviving to term. Samples of blood and ear tissue from each goat were analyzed by Southern blotting in order to detect transgenics and to confirm that mosaic animals did not escape detection. Two animals were identified as transgenic; a female, goat #1, and a male, goat #21. The generation of 2 transgenic animals in 29 goats born from injected embryos represents an integration rate of 7% and can be compared to frequencies previously reported for mouse (10–30%[14]), rabbit (9.5%[7]), pig (10–40%[15]) and sheep (6–10%[16,5]).

At nine months of age, goat #1 was mated to a non-transgenic male. She became pregnant without difficulty and delivered two non-transgenic progeny. A second pregnancy resulted in three additional offspring, one of which was transgenic. Goat #21 (male) was aspermic and was subsequently diagnosed to have a bilateral spermatocele at the head of the epididymis resulting in blockage of normal, developed sperm from entering the vas deferens;

TABLE 1 Summary of injected goat embryos transferred to recipient females.

A.

No. Recipients	Centrifugation	Transfer To	No. Embryos/Stage	No. Pregnant	No. Offspring	No. Transgenic
28	No	Oviduct	137/1-cell 18/2-cell	13[a]	22	2
2	Yes	Oviduct	10/1-cell	0[a]	0	0
5	No	Uterus	31/morula[b]	4	5	0
1	Yes	Uterus	7/morula[b]	1	2	0

B. Efficiency

No. Recipients	No. Embryos	No. Pregnant (%)	No. Offspring (%)	No. Transgenic (%)
36	203	18 (50.0)[c]	29 (14.3)[d]	2 (6.9)[e]

[a]Two of the pregnant animals aborted prematurely.
[b]Embryos injected at the 1-cell stage and cultured for 72 hours.
[c]Percentage is the number of pregnant animals per number of recipients.
[d]Percentage is the number of offspring born per number of embryos transferred.
[e]Percentage is the number of transgenic animals per live births.

a probable congenital defect that is commonly seen in goats. Therefore, we are unclear as to whether this defect relates to the integration of the transgene.

In order to determine the arrangement of the transgene and to estimate the number of copies per cell, blood DNA from the founder female (#1) was digested with three endonucleases and analyzed by Southern blot (Fig. 2). The blot was probed with a 580 bp EcoRI-BglII fragment from the 5′ end of the WAP upstream region labeled to a specific activity of 1×10^8 cpm/μg of DNA by the random hexamer labeling technique[17]. BglII digestion (Lane A) resulted in a predictable 2.9 kb fragment and a 5′ junction fragment approximately 1.0 kb. BglII is known to cut at three sites within the fusion gene (Fig. 1). The lower intensity of the junction fragment indicates that the integrant was inserted at one site and had multiple copies. XbaI (Lane B) cuts once within the fusion gene resulting in a predictable 4.9 kb fragment if the fusion gene integrated in multiple copies and in a tandem array in normal orientation, as well as 5′ junction fragment of approximately 3.3 kb. EcoRI cuts the fusion gene into three fragments (Fig. 1) of which the 3.3 kb fragment will hybridize to the probe on the Southern blot (Lane C).

Figure 3 shows a Southern blot of an EcoRI digest of DNA extracted from blood cells from goat #1 and her five offspring. The blot was probed with a 1.7 kb LAtPA cDNA fragment. The Southern blot shows 3 major bands representing 3.3 kb, 1.1 kb and 472 bp fragments that corresponds to the 5′, 3′, and interior components of the fusion gene respectively. A minor band of 1.6 kb represents the 3′ junction fragment. The probe is weakly homologous to goat DNA. The initial restriction pattern of genomic DNA from the founder female was consistent with the presence of one to two integrated copies of the tPA transgene. However, as shown in Figure 3, the transgenic offspring was shown to contain more copies of the transgene per cell than the founder. This can be interpreted to mean that the founder animal was a mosaic with more copies of the fusion gene per cell than was originally estimated. Additionally, when the blots were counted on a Beta Scope, the data indicated that the bands in offspring 1-3 were twice the value of the #1 founder and supports the concept that the female founder was a mosaic (data not shown). It is estimated from the Southern analysis that the transgenic offspring contained approximately 3-5 copies of the transgene per cell. It should be noted that Southern analysis of DNA from ear tissue gave identical information.

Expression of tPA in milk. The transgenic mother was milked manually twice per day with an average daily yield of 3-4 liters. Milk was stored frozen at −20°C and thawed just prior to analyses. ELISA and amidolytic assays were run on representative milk samples from the first two months of lactation with continued ELISA assay up to the end of lactation (240 days). The daily volume and concentration of LAtPA is shown in Figure 4A. The animal expressed LAtPA at approximately 3 μg/ml during the peak lactation period (1 to 140 days) with an increase in concentration (6.0 μg/ml) toward the end of the lactation period (141 to 240 days). The second lactation produced LAtPA at the same concentration as the first lactation. The first lactation period was truncated from a normal 300 days to 240 days to eliminate the hand milking procedure. The daily milk output and lactation curve were characteristic of a normal dairy goat during her first lactation. The apparent rise in output of LAtPA during the later part of the lactation period did not parallel the constant total protein concentration in the same milk samples. The elevated milk production from September 17 to November 12 corresponds to the initiation of her second estrus season (first

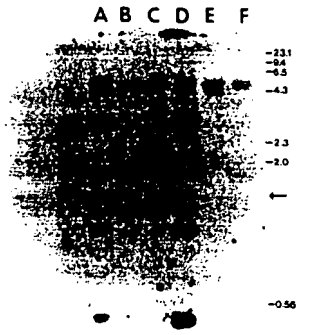

FIGURE 3 Southern blot hybridization of the founder transgenic goat #1 (Lane A) and her five kids (Lanes B, C, D, E, F). DNA isolated from blood was digested with EcoRI, fractionated on an agarose gel, and hybridized to a 1.7 kb LAtPA cDNA fragment. Hybridization of this probe to the transgene shows 3 bands: A: 3.3 kb 5′ junction fragment (Band 1); a 1.1 kb 3′ fragment (Band 2); a 472 bp interior fragment (Band 3). A minor band of 1.6 kb represents the 3′ junction fragment (arrow). Kid #1-3 (Lane D) was shown to be transgenic. Note the intensity of the bands from kid #1-3 was twice as intense as the founder female #1 and indicates that the founder animal is probably a mosaic.

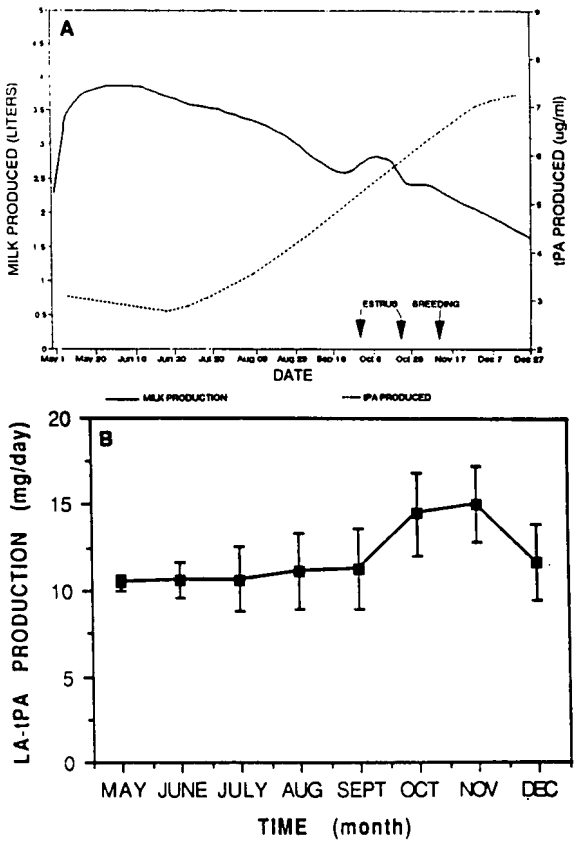

FIGURE 4 (A) LAtPA production throughout lactation in a transgenic goat. The solid line represents the best fit line for daily milk production throughout the 240 day lactation period. The dotted line represents the best fit line for the daily concentration of LAtPA in the milk. The days of her first, second and third estrus cycle and breeding are indicated on the graph. (B) Stability of expression of LAtPA in the milk of a transgenic goat (#1). Milk was harvested and stored as described in the accompanying paper. The concentration of the recombinant enzyme in the milk was determined by ELISA. Total LAtPA produced per day was calculated and daily averages and standard deviations for individual months determined by pooling the data from seven days during the course of each month.

estrus = October 3; second estrus = October 24; third estrus = November 12). The total amount of LAtPA produced per day was calculated and daily averages and standard deviations for individual months are shown in Figure 4B. The output of LAtPA remained relatively constant during the course of lactation, ranging between 11 and 15 mg/day. Although the concentration of the enzyme in milk was relatively low, i.e., approximately 10% of that observed in the recombinant C127 cell line, the continual expression was encouraging since it implied consistent production can be achieved in transgenic animals. The slight rise in output of LAtPA during October and November corresponds to estrus activity. Although the basis for this increase is not known, it is possible that the hormonal changes in the animal associated with the breeding season could have some effect on the expression of the transgene. The female was successfully rebred on November 12 and 13. Interestingly, this period of estrus cyclicity corresponded to a significant rise in the total amount of LAtPA being produced per day. The LAtPA produced in the milk was enzymatically active at approximately 610,000 U/mg. A detailed characterization of the protein is reported in an accompanying paper (Denman et al., *Bio/Technology: This issue*).

These experiments show that targeting transgenes that code for medically important pharmaceutical proteins to the mammary gland of dairy goats is feasible. The level of LAtPA was not high (3 μg/ml) in this first transgenic goat. The likelihood that this goat is mosaic may not allow us to achieve the actual expression level of this gene construct until we generate an F_1 female. However, we have recently produced another female transgenic goat that is producing LAtPA from a β-casein promoter at 2-3 mg/ml (data not shown). At this concentration, the dairy goat may be an economically viable bioreactor for human pharmaceuticals.

EXPERIMENTAL PROTOCOL

Production of transgenic goats. Goats used as donor animals were of either Alpine or Saanen breeds. The timing of estrus was synchronized in the donors with norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, KS; 6 mg). Prostaglandin was administered after the first 7-9 days to remove endogenous sources of progesterone. At day 13 following progesterone administration, follicle-stimulating hormone (FSH, Schering Corp., Kenilworth, NJ) was given to goats at a dose of 18 mg over three days in twice daily injections (Warren Foote, personal communication). During the anestrus season (after February), the dose of FSH was increased to 24 mg administered similarly over three days in twice daily injections. Twenty-four hours following implant removal, the donor animals were mated several times to fertile males over a two-day period. Recipient animals were synchronized by the same protocols as the donor animals except that a single non-superovulatory injection of pregnant mares serum gonadotropin (PMSG, Sigma, St. Louis, MO) was given on day 13 of progesterone treatment in place of the FSH. From September to January, the recipients received 400 IU PMSG, and from February to April they received 750 IU PMSG. Recipient females were mated to vasectomized males to ensure estrus synchrony. Seventy-two hours following implant removal, embryos were recovered surgically from the oviducts of donors. Embryos were flushed from oviducts associated with ovulated ovaries through a cannula with sterile phosphate-buffered saline and were collected in a petri dish as previously reported[15]. The HindIII-BamHI fragment of WAP-LAtPA was injected into one of the two pronuclei from one-cell embryos or into a nucleus of one blastomere of two-cell embryos at a concentration of 1 μg/ml. Embryos were surgically transferred into the oviducts of the recipient females or to the uteri following a 72 hour culture period.

Identification of transgenic goats. DNA was extracted from the buffy coat recovered from blood of goat #1. Following digestion with restriction enzymes as indicated in the legend to Figure 1, DNA was fractionated and blotted onto nitrocellulose[18]. The probe was a 1.7 kb LAtPA cDNA isolated from the region of the whey acid protein gene 2600 bp upstream of the transcriptional start site[3]. The probe was radioactively labeled by the random primer method[17].

Enzyme activity and protein assays. Plasminogen activator concentrations (amidolytic activity) were determined with an indirect method using the plasmin substrate Val-Leu-Lys-*p*-nitroanilide[12] (S-2251, Helena Labs, Inc.). LAtPA concentration was estimated using the Imubind® tPA ELISA assay kit (American Diagnostics, Chicago, IL) adapted to determine LAtPA in goat's milk.

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References

1. Hennighausen, L. 1990. The mammary gland as a bioreactor: production of foreign proteins in milk. Protein Expression and Purification 1:3–8.
2. Ebert, K. M. and Selgrath, J. P. 1991. Changes in domestic livestock through genetic engineering. *In:* Applications of Basic Research in Mammalian Development. Pedersen, R. A., McLaren, A. and First, N. (Eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. Gordon, K., Lee, E., Vitale, J. A., Smith, A. E., Westphal, H. and Henninghausen, L. 1987. Production of human tissue plasminogen activator in transgenic mice. Bio/Technology 5:1183–1187.
4. Pittius, C. W., Henninghausen, L., Lee, E., Westphal, H., Nicols, E., Vitale, J. and Gordon, K. 1988. A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice. Proc. Natl. Acad. Sci. USA 85:5874–5878.
5. Clark, A. J., Bessos, H., Bishop, J. O., Brown, P., Harris, S., Lathe, R., McClenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B. A. and Wilmut, I. 1989. Expression of human anti-hemophilic factor IX in the milk of transgenic sheep. Bio/Technology 7:487–492.
6. Yu, S. H., Deen, K. C., Lee, E., Hennighausen, L., Sweet, R. W., Rosenberg, M. and Westphal, H. 1989. Functional human CD4 protein produced in milk of transgenic mice. Mol. Biol. Med. 6:255–261.
7. Buhler, Th. A., Bruyere, Th., Went, D. F., Stranzinger, G. and Burki, K. 1990. Rabbit β-casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits. Bio/Technology 8:140–143.
8. Archibald, A. L., McClenaghan, M., Hornsey, V., Simons, J. P. and Clark, A. J. 1990. High-level expression of biologically active human α_1-antitrypsin in the milk of transgenic mice. Proc. Natl. Acad. Sci. USA 87:5178–5182.
9. Meade, H., Gates, L., Lacy, E. and Lonberg, N. 1990. Bovine alpha S1-casein gene sequences direct high level expression of active human urokinase in mouse milk. Bio/Technology 8:443–446.
10. Wall, R. J., Pursel, V. G., Shamay, A., McKnight, R. A., Pittius, C. W. and Henninghausen, L. 1991. High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. Proc. Natl. Acad. Sci. USA 88:1696–1700.
11. Simos, E., Voutsinas, L. P. and Pappas, C. P. 1991. Composition of milk of native Greek goats in the region of Metsovo. Small Ruminant Research 4:47–60.
12. Lau, D., Kuzma, G., Wei, C. M., Livingstron, D. J., and Hsiung, N. 1987. A modified human tissue plasminogen activator with extended half-life *in vivo*. Bio/Technology 5:953–958.
13. Selgrath, J. P., Memon, M. A., Smith, T. E. and Ebert, K. M. 1990. Collection and transfer of microinjectable embryos from dairy goats. Theriogenology 34:1195–1205.
14. Cuthbertson, R. A. and Klintworth, G. K. 1988. Biology of Disease: Transgenic mice - a gold mine for furthering knowledge in pathobiology. Laboratory Investigation 58:484–502.
15. Ebert, K. M., Smith, T. E., Buonomo, F. C., Overstrom, E. W. and Low, M. J. 1990. Porcine growth hormone gene expression from viral promoters in transgenic swine. Animal Biotechnology 1:145–159.
16. Hammer, R. E., Pursel, V. G., Rexroad, C. E., Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D., and Brinster, R. L. 1985. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683.
17. Feinberg, A. and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 72:248–254.
18. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. 77:5201–5205.
19. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

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TRANSGENIC CORNUCOPIA: PLUMS, CARNATIONS, AND MELONS

A NEW BACULOVIRUS INSECTICIDE

HIGH LEVEL EXPRESSION OF ACTIVE HUMAN ALPHA-1-ANTITRYPSIN IN THE MILK OF TRANSGENIC SHEEP

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We describe the generation of five sheep transgenic for a fusion of the ovine β-lactoglobulin gene promotor to the human α_1-antitrypsin ($h\alpha_1AT$) genomic sequences. Four of these animals are female and one male. Analysis of the expression of $h\alpha_1AT$ in the milk of three of these females shows that all express the human protein at levels greater than 1 gram per liter. In one case initial levels exceeded 60 grams per liter and stabilized at approximately 35 grams per liter as lactation progressed. Human α_1AT purified from the milk of these animals appears to be fully N-glycosylated and has a biological activity indistinguishable from human plasma-derived material.

The prospect of producing large quantities of therapeutic proteins in the milk of transgenic livestock was publically raised some years ago[1,2]. As an alternative to cell culture systems, this production route is appealing because of the simplicity of access to the expressed protein, the high production capabilities of the mammary gland, the relatively low operating costs, and finally, the potentially unlimited expansion of the producer animals through established and emerging methods of animal husbandry. Like expression systems based on cultured mammalian cells, the mammary gland appears to be capable of performing the post-translational modifications vital to the activity or stability of certain pharmacologically active human proteins. In the last five years numerous publications have appeared attesting to the feasibility of this approach (for review, see ref. 3). Typically the gene of interest (either cDNA or genomic DNA) is fused to the regulatory sequences of the gene for a milk protein, and the fusion construct used to generate transgenic animals. In numerous cases the desired protein has been found in the milk, however the yields of protein have been extremely variable and usually much less than 1 gram per liter, although levels as high as 23 grams per liter in mice[4] and 2 grams per liter in pigs[5] have been obtained with foreign milk proteins, and 1–2 grams per liter of human urokinase, a plasminogen activator, has been produced in mouse milk[6].

Human α_1-antitrypsin ($h\alpha_1AT$) is a 394 amino acid glycoprotein which is normally present at 2 grams per liter in plasma[7]. The primary site of $h\alpha_1AT$ production in the body is the liver[7–9], and genetic deficiencies in circulating concentrations of $h\alpha_1AT$ are one of the most common lethal hereditary disorders to affect Caucasian males of European descent and sufferers are at risk of developing life-threatening emphysema. Replacement therapy using human plasma-derived α_1AT, has been sanctioned in the USA[7] where the large number (>20,000) of affected individuals and large amounts needed (~200 grams/patient/year[10]) make a strong case for an alternative, recombinant DNA-derived source, which is capable of yielding large quantities of $h\alpha_1AT$ and of performing the glycosylation events needed for plasma stability[10]. Recently Archibald et al.[11] reported yields of up to 7 grams per liter of biologically active $h\alpha_1AT$ in the milk of transgenic mice expressing a minigene containing the sheep betalactoglobulin (BLG) promotor fused to $h\alpha_1AT$ sequences, which comprise part of exon 1 and the remaining, downstream introns and exons, excluding intron 1. If similar high yields could be obtained in the milk of transgenic livestock, this could form the basis for a manufacturing process.

In this paper we demonstrate that the sheep mammary gland can offer a production route for large quantities of glycosylated, bioactive $h\alpha_1AT$. Using the $h\alpha_1AT$ minigene described above, we report the generation, lactation, and milk protein analysis of three founder transgenic animals. Two of the animals produce 1–5 grams per liter quantities of $h\alpha_1AT$ whilst the third produces ~35 grams per liter making $h\alpha_1AT$ the major protein in the milk. These levels have been sustained in all cases through 7 weeks of lactation and exceed by several orders of magnitude previously reported yields of foreign proteins in sheep milk and, in one case, the yield is substantially (>17 fold) higher than that reported for any foreign, non-milk protein in any transgenic system[3]. We believe these results confirm the feasibility of using mammals as bioreactors for the production of human therapeutic proteins.

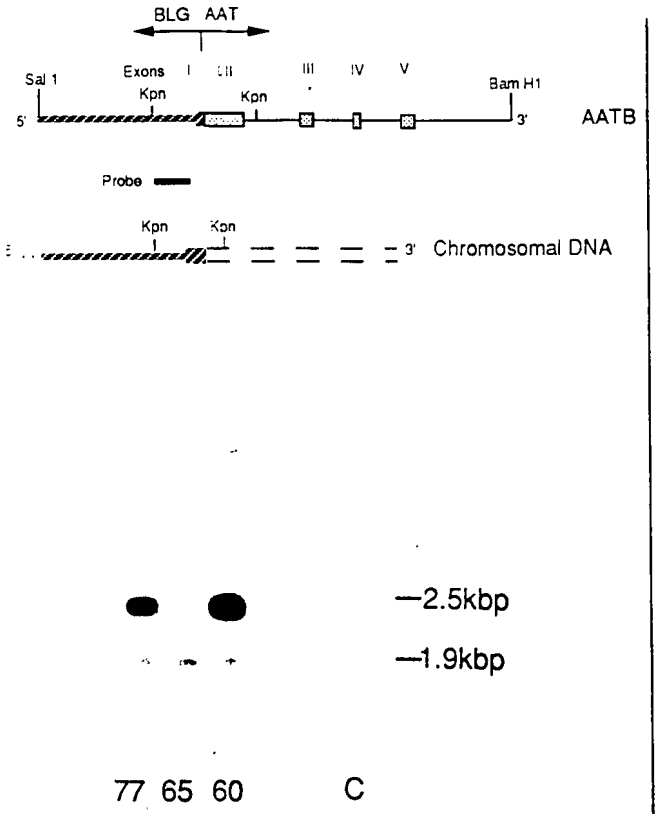

FIGURE 1 Southern blot analysis of transgenic DNA. The diagram shows the relevant region of the sheep chromosomal BLG locus as well as the intron/exon structure of the AATB construct used for injection (also see ref. 11); hatched regions correspond to BLG sequences and stippled regions and plain lines correspond to AAT sequences. DNA purified from the peripheral blood lymphocytes of transgenic sheep 60, 65 and 77 and a non-transgenic control animal (C) was digested with KpnI and analyzed as described in the Experimental Protocol. The membrane was hybridized with a radioactive probe homologous to 800 bp of the ovine BLG promoter (see diagram) and was generated by random priming (Stratagene). This reveals a 2.5 kbp internal band from intact transgenes and a 1.9 kbp band derived from endogenous BLG sequences (see diagram).

TABLE 1 Summary of the generation of transgenic sheep

Parameter	Value
No. eggs injected	549
No. eggs surviving	439
No. recipients	152
No. eggs per recipient	2.88
No. of pregnancies	73
No. births	113
No. screened	112*
No. transgenic	5
Percent of births transgenic	4.5
Percent of injected eggs transgenic	0.91

*One animal was stillborn and proved unsuitable for analysis.

RESULTS

Generation of transgenic sheep. Archibald et al.[11] recently described the production of bioactive $h\alpha_1AT$ in the milk of mice transgenic for a hybrid ovine BLG-$h\alpha_1AT$ gene, referred to as AATB. Of seven lines of animals expressing the AATB transgene at variable levels in the lactating mammary gland, four produced milk levels of greater than 0.5 grams per liter $h\alpha_1AT$ with one yielding a level in excess of 7 grams per liter. For this study, we made use of the same hybrid AATB construct.

Initially, we extended the observations of Archibald and colleagues by generating nine G_0 founder mice transgenic for the AATB fusion. Although levels of $h\alpha_1AT$ produced in the milk of these animals varies from line to line, all express the transgene at between 0.4 mg and 12.45 grams per liter. Moreover, the highest expressing animal has transmitted the transgene to her offspring and all G_1 females (three) exhibit a capacity similar to that of their mother to secrete $h\alpha_1AT$ with their milk (unpublished data).

These studies suggest that the AATB construct is efficient at directing the expression of $h\alpha_1AT$ to the lactating mammary gland with concomitant secretion of the human protein. To confirm that this is true not only in mice but also in sheep, we generated sheep transgenic for the AATB fusion gene. A total of 549 sheep eggs were microinjected with purified AATB DNA giving rise to 113 lambs (Table 1). One of these animals was stillborn and proved unsuitable for further analysis. Of the remaining 112, five proved to be positive for the AATB hybrid gene upon Southern blot analysis of genomic DNA samples. Four of these are female and one male.

These five animals developed normally and have shown no ill effects attributable to the presence of the transgene. To date, three of the females (nos. 60, 65 and 77) have produced offspring. Sheep 60 produced two female lambs, one of which is transgenic, sheep 65 produced one non-transgenic male and sheep 77 produced one non-transgenic female (data not shown). To assess the integrity of the incorporated transgenes in these three G_0 animals we performed Southern blot analyses of genomic DNA derived from peripheral blood lymphocytes. Cleavage of integrated copies with KpnI should release an internal fragment of 2.5 kbp (Fig. 1). This is revealed with a probe covering the first 800 bp of the BLG sequences present in AATB. The probe also reveals a 1.9 kbp band derived from endogenous ovine BLG sequences by hybridization to identical target sequences. Comparisons of band intensities with copy number controls (data not shown) and the endogenous BLG bands suggest that sheep 60 contains ~10 copies of the transgene, sheep 65 ~2 copies and sheep 77 ~5 copies. Analyses using other restriction enzymes and probes suggest that the multiple integrants contain intact copies of the transgene (data not presented). However, as previously found in transgenic sheep[12], the arrays are complex with both head to head and head to tail repeats. The elucidation of the exact structure of these arrays awaits further study.

Levels of human α_1AT in transgenic sheep milk. The offspring from animals 60, 65 and 77 were artificially reared and milk collected daily from their lactating mothers. Samples were pooled on a weekly basis and analyzed for the presence of $h\alpha_1AT$. Initial determinations were performed with both a radial immunodifusion assay (RID) and ELISA. Neither of these techniques produce a cross-reaction with sheep α_1AT. A good correlation was observed between results obtained with these techniques and further determinations were performed using RID alone.

Levels of $h\alpha_1AT$ present in the milk of all three founder animals have exceeded 1 gram per liter (Table 2). There is no direct relationship between transgene copy number and levels of expression. It is notable, however, that yields do increase with increasing copy number. Sheep 60 produced 63 grams per liter $h\alpha_1AT$ in week one but has since stablized to yield ~35 grams per liter in

subsequent weeks. The human protein is consistently ~50% of the total protein in the milk of this animal. Sheep 65 produced 3.8 grams per liter in week one and has since stablized at around 1.5 grams per liter. Again this is a constant percentage of the total protein produced of about 3.5%. In contrast, Sheep 77 began secreting $h\alpha_1AT$ at 0.9 grams per liter and has since increased output attaining 3.5 grams per liter in week seven. This reflects an increase in the percentage of total protein that is $h\alpha_1AT$ from 1.4 to 10%. We have no explanation for this at present.

It should be noted that milk from week one contained colostrum and as such had higher concentrations of both $h\alpha_1AT$ and total protein. However, the total protein levels recorded for subsequent weeks has remained within observed limits for sheep milk despite being higher than expected for this breed (Blackface/Friesland). We are, therefore, not in a position to comment on whether endogenous protein production has been suppressed in these animals or whether total protein production has been increased.

Characterization of human α_1AT from transgenic sheep milk. Milk from founder animals 65, 77 and 60 was analyzed by SDS/PAGE (Fig. 2, lanes 2, 3 and 4 respectively). A novel band of apparent 54 kD molecular weight was observed in all three samples (indicated by arrow). This is the predicted molecular weight of native plasma derived $h\alpha_1AT$ (Fig. 2, lane 8). We confirmed this to be $h\alpha_1AT$ by western blotting (data not shown). Note that in the sample derived from sheep 60, the $h\alpha_1AT$ is the major protein in the milk.

Milk samples from all three sheep were defatted and $h\alpha_1AT$ was purified from the remaining material using anion exchange, dye affinity, hydrophobic interaction and gel filtration chromatography (manuscript in preparation). When analyzed by reducing (data not shown) and non-reducing SDS/PAGE (Fig. 2, lanes 5, 6 and 7) all three products migrate as a single band of about 54 kD similar to that observed for plasma derived $h\alpha_1AT$ (Fig. 2, lane 8). We estimate the purity of the three products to be >95% following silver staining, densitometry scanning and HPLC analysis (data not shown). Sheep milk naturally contains 1–2 μg per ml α_1AT. Our purified material could therefore contain a small percentage of sheep α_1AT that would not be revealed by our RID or ELISA assays, which are specific for the human protein. However, a comparison of the results obtained from these two techniques with total protein estimates indicated that our purified $h\alpha_1AT$ is at least 95% human protein. This is supported by amino terminal sequence data, which do not reveal any contamination with sheep α_1AT (manuscript in preparation).

Glycosylation of human α_1AT from transgenic sheep milk. Human α_1AT has three N-linked branched carbohydrate chains linked to asparagines 46, 83 and 247. Non-glycosylated recombinant $h\alpha_1AT$ is active but exhibits an accelerated *in vivo* plasma clearance[10], probably reflecting the absence of carbohydrate moieties. The apparent molecular weight of the material purified from transgenic sheep milk suggests that it is fully glycosylated, and to determine if this is so samples were cleaved with N-glycosidase F (Fig. 3). Lanes 1–4 contain uncleaved material and lanes 5–8 cleaved samples. In all cases, the $h\alpha_1AT$ purified from transgenic sheep milk behaves similarly to $h\alpha_1AT$ purified from human plasma (Fig. 3, lanes 1 and 8). Digestion of all samples results in a shift of electrophoretic mobility similar to that observed with human plasma derived α_1AT (Fig. 3, lanes 1 and 8). Furthermore, all of our purified material appears to be fully N-glycosylated (compare lanes 2–4 with lanes 6–8). Re-examination of the $h\alpha_1AT$ from complete milk (eg.

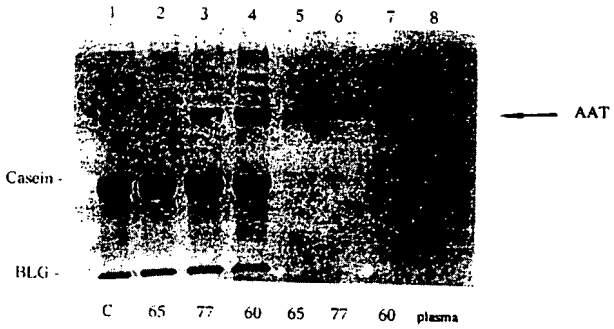

FIGURE 2 Non-reducing SDS-PAGE of transgenic sheep milk and purified $h\alpha_1AT$. Aliquots (0.1 μl) of whole milk from transgenic sheep 65, 77, 60 and a control (C) non-transgenic animal (lanes 1–4) or 1 μg of $h\alpha_1AT$ purified from the milk of transgenic sheep (lanes 5–7) were analyzed on a 12% non-reducing, SDS-PAGE gel as described in the Experimental Protocol. Lane 8 contains 1 μg $h\alpha_1AT$ purified from human plasma (Miles, Inc.). The running positions of casein and betalactoglobulin are indicated on the left of the figure. The position of plasma derived $h\alpha_1AT$ is indicated by the arrow on the right of the figure.

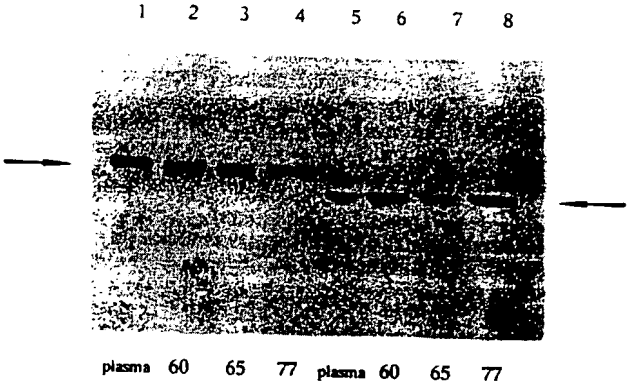

FIGURE 3 SDS-PAGE of glycosylated and deglycosylated $h\alpha_1AT$. $h\alpha_1AT$ (0.5 μg) samples purified from human plasma (Miles, Inc.) or from the milk of transgenic sheep 60, 65 and 77 were treated with (lanes 5–8) or without (lanes 1–4) N-glycosidase F as described in the Experimental Protocol. The arrow to the left of the figure indicates the position of glycosylated material (54 kD), the arrow to the right indicates the position of cleaved deglycosylated material (45 kD). MW markers are not shown.

TABLE 2 Analysis of human α_1AT in transgenic sheep milk.

Sheep	Week	Protein Content	h α_1AT Content
60	1	127.2	63.0
	4	66.8	31.7
	7	71.2	37.5
65	1	72.4	3.8
	4	44.1	1.3
	7	41.6	1.5
77	1	64.0	0.9
	4	44.4	2.2
	7	35.8	3.5

Animals were milked daily and the weekly produce was pooled prior to analysis. Figures from three representative weeks are presented in grams per liter of milk.

track 4, Fig. 2) shows that it corresponds in mobility to the glycosylated products shown in Figure 3 and therefore excludes the possibility that the purification was selective for glycosylated forms of α_1AT, indicating that all the hα_1AT is glycosylated. These results demonstrate the ability of the ovine mammary gland to N-glycosylate large quantities of secreted protein. We are currently determining the nature of these sugar moieties.

Bioactivity of hα_1AT purified from transgenic sheep milk. To analyze the activity of our purified hα_1AT we compared its ability to inhibit trypsin to that of two samples of plasma-derived hα_1AT using a colorimetric assay. A standard curve generated with one plasma-derived source was used to determine the activities of each of the other samples. In all cases, the hα_1AT purified from transgenic sheep milk shows similar activity to both plasma-derived products (Table 3).

DISCUSSION

We report in this paper the production in sheep milk of large amounts of a foreign protein, human α_1AT. We presume that this protein is made predominantly in the mammary gland for several reasons. First, analysis of the tissue-specificity of transcription from the AATB construct in mice indicates that the major site of transcription is the mammary gland, although in some animals a low level of expression from the salivary gland was noticed[11]. Second, if hα_1AT were synthesized outside the mammary gland, it would presumably gain access to the mammary gland via the blood. However circulating levels of hα_1AT are negligible compared to sheep α_1AT whereas this situation is reversed in the milk (data not shown). Direct analysis of RNA expression will be performed eventually, however at present we are concerned not to compromise the animals during their first lactation.

Concentrations up to 35 grams per liter of hα_1AT have been obtained. This level of hα_1AT production has now been sustained throughout the lactation period (twelve weeks); this situation contrasts with that recently reported for transgenic swine producing mouse whey acid protein where lactation itself was not sustained in 2 out of 3 lines as a result of transgene expression[5]. With milk yields per lactation ranging from 250–800 liters, according to sheep breed, the overall yield of hα_1AT per animal per lactation could exceed 10 kg. The hα_1AT recovered shows similar bioactivity to the human plasma-derived product. In addition, the hα_1AT produced by all three animals is fully N-glycosylated and we are presently investigating the exact sugar composition of the carbohydrate side chains. This demonstrates that in the mammary gland, the glycosylation apparatus has not been saturated by the requirement for hα_1AT glycosylation even though normally only a small proportion of the endogenous milk protein (<10%) is glycosylated and most of this represents O-linked glycosylation of κ-casein. Despite the commandeering of the animals' transcriptional and translational machinery for foreign protein production, all the transgenic sheep described here are perfectly normal and healthy. Although we have so far only been able to demonstrate transgene transmission in one of the three female sheep [the one transgenic male, has transmitted the transgene (data not shown)], the seven out of eight transgenic sheep previously generated by Clark and colleagues[12] have been found to transmit their transgenes in an unrearranged fashion (J. Clark, personal communication).

With few exceptions[13–15] it still remains the case that expression from the same transgene construct is highly variable between different lines. This has been attributed to various causes, including host genetic background, site(s) of chromosomal insertion, absence of certain transcriptional elements, etc[16]. Although there is no formal proof, we believe that the sheep BLG gene used to provide control elements for our transgene constructs has all the regulatory sequences necessary to confer high expression on a foreign gene fragment, since expression of the complete BLG gene in transgenic mice led to a range of yields but nearly all of them were high[4]. Although dramatically lower expression levels have been reported for fusion constructs between foreign genes and milk protein gene promotors including sheep BLG, this may be attributable in part to the absence of native introns in the foreign gene inserts[17]. Improvements in expression have been obtained when native, foreign or hybrid introns are added back[17–19]. When originally expressed in mice by Archibald et al.[11], the minigene used in our study gave hα_1AT yields of 80 milligrams −7.7 grams per liter with some animals not producing any detectable protein at all. Repeating this work we obtained a range of yields from 0.4 milligrams −12.45 grams per liter from nine different lines, a 30,000-fold range in variation. Although only 3 founder ewes have been analyzed in the study reported here (the fourth ewe is about to give birth), a 10-fold range of yields was obtained. While a comparison of the mice and sheep hα_1AT yields is questionable due to the small sample size, it is notable that the expression levels in sheep are on average higher and less variable. This may be a consequence of the homologous combination of an introduced sheep milk protein gene promotor operating in a sheep mammary gland environment.

In summary, we describe the production of high levels of a human therapeutic protein, α_1AT, in sheep milk. In one case the hα_1AT represents nearly 50% of total milk protein throughout the lactation period. These results indicate that it is possible to dramatically alter milk composition, opening up opportunities in the dairy industry to carry out a range of manipulations from over-expression of existing proteins to the introduction of novel milk proteins, which may allow improvements in milk formulations for both adult and infant consumption. In addition, this level of hα_1AT production exceeds those obtained in bacteria (15% total cell protein[20,21]), yeast (40% soluble protein[22]), and cultured mammalian cells (<1mg/10^6 cells/24h[23]), and provides a strong impetus to the further exploitation of transgenic sheep as bioreactors for the production of large amounts of pharmacologically active proteins.

TABLE 3 Bioactivity of hα_1AT purified from transgenic sheep milk.

Source of Purified hα_1AT	Percent Activity
Sheep 60 milk	95 ± 12
Sheep 65 milk	93 ± 22
Sheep 77 milk	86 ± 15
Human plasma α1AT (Miles, Inc.)	100
Human plasma α1AT (Sigma)	94 ± 5

Results are derived from 5 separate assays performed on two separate days and are normalized to the values obtained with the Miles, Inc. sample.

EXPERIMENTAL PROTOCOL

Generation of transgenic sheep. Transgenic sheep were generated essentially as described by Simons et al.[12] with the following differences: superovulation was induced with regimes of equine, porcine or ovine FSH; ovulation was synchronized in donor ewes (Scottish Blackface) using Receptal (Hoechst Animal Health); eggs were collected from donor ewes artificially inseminated with approximately 10^7 fresh, motile spermatozoa (Friesland) by intrauterine laparoscopy; eggs were collected by mid-ventral laparoptomy

approximately 17 hours after the expected mode time of ovulation.

DNA preparation and analysis. Peripheral blood lymphocytes were prepared from transgenic sheep blood using Histopaque (Sigma) according to the manufacturers instructions. Genomic DNA was prepared by proteinase K (BCL) digestion and phenol extraction. Following digestion with appropriate restriction enzyme(s), samples were subjected to electrophoresis in 1% agarose gels, transferred to Duralon trademark membranes and hybridized to radioactive probes as described by the manufacturer (Stratagene).

Gel analysis of protein samples. Milk and purified $h\alpha_1AT$ samples were diluted in 75 mM-Tris/HCl buffer at pH 6.8, containing 2.5% (w/v) SDS and 10% (w/v) glycerol. These samples were boiled for 2 minutes and then electrophoresed on 12% discontinuous SDS polyacrylamide gels[24]. After running, gels were stained with 0.125% Coomassie blue R-250 in a 50% methanol/10% acetic acid solution and destained with the solvent alone.

Measurement of human α_1AT. Concentrations of $h\alpha_1AT$ were measured by radial immunodiffusion (RID) and confirmed by enzyme linked immunosorbant assay (ELISA). RID plates were obtained from the Binding Site, and $h\alpha_1AT$ levels were measured using the method described in the manufacturer's instructions. For the ELISA assay, polyclonal rabbit anti-human $h\alpha_1AT$ antibodies (Dako) were diluted 1/1000 in 0.1 M-$NaHCO_3$, pH 8.2. Aliquots (250 µl) of this solution were added to each well of microtiter plates and then these were incubated overnight. The next day the plates were washed, and various sample dilutions in 1/1000 normal rabbit IgG (Dako) were added to the wells. The plates were incubated for 2.5 hours before adding 1/1000 diluted biotinylated polyclonal rabbit anti-human $h\alpha_1AT$ second antibodies. The plates were then incubated for 90 minutes before being washed and adding a streptavidin/biotin-horseradish peroxidase conjugate (Boehringer). This was followed by another 90 minute incubation before the plate was again washed and finally 100 µl of the substrate solution 1,2 phenylenediamine and 0.01% (v/v) hydrogen peroxide was added. Color was allowed to develop and this was measured at 492 nm. The $h\alpha_1AT$ content of samples was measured by comparing the 492 nm results to those obtained with standards containing known amounts of $h\alpha_1AT$. Except for the initial step where the antibodies were bound to the plate, all washes were carried out, and dilutions made, in phosphate buffered saline containing 5% bovine skimmed milk; 0.1% Tween 20; and 0.1 mM-EDTA. All incubations were at room temperature in a moisture chamber. The second antibodies were biotinylated by incubating them in 50 mM-Tris/acetate buffer at pH 7.5 for 4 hours with 0.8 mM biotinyl-ε-amino caproic acid N-hydroxysuccinimide ester and then dialyzing against the above buffer alone. Plasma derived samples of $h\alpha_1AT$ were purchased from Sigma or were the kind gift of Miles, Inc. (Berkeley, CA).

Deglycosylation of human α_1AT. Samples of purified $h\alpha_1AT$ (0.5 mg/ml) were suspended in 100 mM-sodium phosphate buffer at pH 7.5 containing: 25 mM-EDTA; 1% *n*-octylglucoside; 1% 2-mercaptoethanol; and 0.1% SDS. These were boiled for 5 minutes, cooled to room temperature, and then 10 units/ml of N-glycosidase F (Boehringer) was added. Samples were then incubated for 16 hours at room temperature before they were run on SDS-PAGE. Control samples of $h\alpha_1AT$ were treated in exactly the same way, except no N-glycosidase was added.

Bioassay of human α_1AT. The $h\alpha_1AT$ activity assay used is based on the affinity of human α_1AT for trypsin. Nα-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), in the presence of trypsin (Sigma), breaks down to benzoylarginine and the colored compound *p*-nitroanalide, the absorbance of which can be measured at 405 nm. Dilutions of purified $h\alpha_1AT$ (130 µl) were incubated at room temperature with 50 µl of 0.25 mg/ml porcine trypsin type II (Sigma) in 50 mM-Tris/acetate buffer at pH 7.5 for 5 minutes before the addition of 20 µl of the chromogenic substrate BAPNA (Sigma). After 15 minutes the absorbances at 405 nm were read. A standard curve using constant amounts of trypsin (62.5 µg/ml) and BAPNA (1.5 mM), and varying amounts of human plasma-derived α_1AT (5–40 µg/ml; Sigma) was constructed. The inhibition of the same amount of trypsin/BAPNA by purified $h\alpha_1AT$ samples was then translated into a percentage activity relative to the purified plasma-derived $h\alpha_1AT$ (Miles, Inc.).

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Note added in proof: The fourth ewe transgenic for AATB has given birth to a female lamb that has inherited the transgene. The mother is expressing $h\alpha_1AT$ in her milk at a level of 3.2 g/l, which further reinforces our view that high level expression from this construction in the milk of transgenic sheep is the norm rather than the exception.

References

1. Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C., and Evans, R. M. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature **300**:611–615.
2. Lovell-Badge, R. H. 1985. New advances in the field. Nature **315**:628–629.
3. Hennighausen, L., Ruiz, L., and Wall, R. 1990. Transgenic animals-production of foreign proteins in milk. Curr. Op. in Biotech. **1**:74–78.
4. Simons, J. P., McClenaghan, M., and Clark, A. J. 1987. Alteration of the quality of milk by expression of sheep β-lactoglobulin in transgenic mice. Nature **328**:530–532.
5. Wall, R. J., Pursel, V. G., Shamay, A., McKnight, R. A., Pittius, C. W., and Hennighausen, L. 1991. High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. Proc. Natl. Acad. Sci. USA **88**:1696–1700.
6. Meade, H., Gates, L., Lacy, E., and Lonberg, N. 1990. Bovine αS_1-casein gene sequences direct high level expression of active human urokinase in mouse milk. Bio/Technology **8**:443–446.
7. Crystal, R. G. 1989. The α1-antitrypsin gene and its deficiency states. Trends in Genet. **5**:411–417.
8. Carlson, J. A., Rogers, B. B., Sifers, R. N., Hawkins, H. K., Finegold, M. J., and Woo, S. L. C. 1988. Multiple tissues express α1-antitrypsin in transgenic mice and man. J. Clin. Invest. **82**:26–36.
9. Kelsey, G. D., Povey, S., Bygrave, A. E., and Lovell-Badge, R. H. 1987. Species- and tissue-specific expression of human α1-antitrypsin in transgenic mice. Genes and Development **1**:161–171.
10. Casolaro, M. A., Fells, G., Wewers, M., Pierce, J. E., Ogushi, F., Hubbard, R., Sellers, S., Forstrom, J., Lyons, D., Kawasaki, G., and Crystal, R. G. 1987. Augmentation of lung antineutrophil elastase capacity with recombinant human α1-antitrypsin. J. Appl. Physiol. **63**:2015–2023.
11. Archibald, A. L., McClenaghan, M., Hornsey, V., Simons, J. P., and Clark, A. J. 1990. High-level expression of biologically active human α1-antitrypsin in the milk of transgenic mice. Proc. Natl. Acad. Sci. USA **87**:5178–5182.
12. Simons, J. P., Wilmut, I., Clark, A. J., Archibald, A. L., Bishop, J. O., and Lathe, R. 1988. Gene transfer into sheep. Bio/Technology **6**:179–183.
13. Grosveld, F., van Assendelft, G. B., Greaves, D. R., and Kollias, G. 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51**:975–985.
14. Greaves, D. R., Wilson, F. D., Lang, G., and Kioussis, D. 1989. Human CD2 3′-flanking sequences confer high-level, T cell-specific, position-independant gene expression in transgenic mice. Cell **56**:979–986.
15. Bonifer, C., Vidal, M., Grosveld, F., and Sippel, A. 1990. Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice. EMBO J. **9**:2843–2848.
16. Bishop, J. O. and Al-Shawi, R. 1989. Gene expression in transgenic animals, p. 249–260. *In:* Evolution and Animal Breeding. Hill, W. G. and Mackay, T. F. C., (Eds.). CAB International, Wallingford, UK.
17. Brinster, R. L., Allen, J. M., Behringer, R. R., Gelinas, R. E., and Palmiter, R. D. 1988. Introns increase transcriptional efficiency in transgenic mice. Proc. Natl. Acad. Sci. USA **85**:836–840.
18. Palmiter, R. D., Sandgren, E. P., Avarbock, M. R., Allen, D. D., and Brinster, R. L. 1991. Heterologous introns can enhance expression of transgenes in mice. Proc. Natl. Acad. Sci. USA **88**:478–482.
19. Choi, T., Huang, M., Gorman, C., and Jaenisch, R. 1991. A generic intron increases gene expression in transgenic mice. Mol. Cell. Biol. **11**:3070–3074.
20. Courtney, M., Buchwalder, A., Tessier, L-H., Jaye, M., Benavente, A., Balland, A., Kohli, V., Lathe, R., Tolstoshev, P., and Lecocq, J-P. 1984. High-level production of biologically active human α1-antitrypsin in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81**:669–673.
21. Garver, R. I., Chytil, A., Karlsson, S., Fells, G. A., Brantly, M. L., Courtney, M., Kantoff, P. W., Nienhuis, A. W., French Anderson, W., and Crystal, R. G. 1987. Production of glycosylated physiologically "normal" human α1-antitrypsin by mouse fibroblasts modified by insertion of a human α1-antitrypsin cDNA using a retroviral vector. Proc. Natl. Acad. Sci. USA **84**:1050–1054.
22. Sleep, D., Belfield, G. P., Ballance, D. J., Steven, J., Jones, S., Evans, L. R., Moir, P. D., and Goody, A. R. 1991. *Saccharomyces cerevisiae* strains that overexpress heterologous proteins. Bio/Technology **9**:183–187.
23. Pavironi, A., Skern, T., Le Meur, M., Lutz, Y., Lathe, R., Crystal, R. G., Fuchs, J-P., Gerlinger, P., and Courtney, M. 1989. Recombinant proteins of therapeutic interest expressed by lymphoid cell lines derived from transgenic mice. Bio/Technology **7**:1049–1054.
24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277**:680–685.

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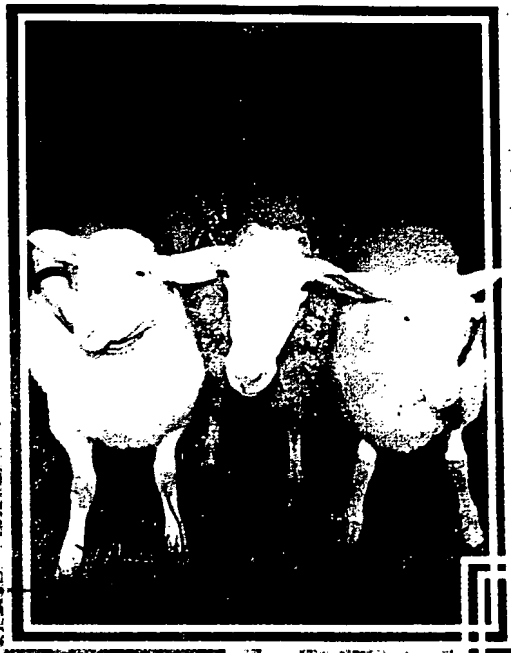

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(5)

GENE TRANSFER INTO SHEEP

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Gene transfer into animals has considerable potential for livestock improvement. If this potential is to be realized, the ease of generation of transgenic livestock will be of major importance. We report here the production of six transgenic sheep by microinjection of DNA into early embryos (1.2% of embryos transferred). Three different gene constructs were injected and transgenic sheep were obtained with each. The transgenic animals have all incorporated the DNA without detectable rearrangement, and where multiple copies were integrated, they are present in arrays of tandem repeats. Transmission of transferred genes to progeny of three of the sheep has been demonstrated. Five founder transgenic sheep described carry genes designed to direct the production of human clotting factor IX or human α1-antitrypsin in milk. Transgenic animals carrying such genes may ultimately provide a new source of these and other therapeutic proteins.

Gene transfer into the mouse germline is now a routine procedure[1]. Typically, single-cell eggs are microinjected with DNA into one pronucleus, and are implanted into a foster mother. A proportion of the animals which develop from these eggs carry the injected DNA integrated into a chromosome. The foreign genes (transgenes) are often expressed correctly and can have profound phenotypic effects on transgenic mice, exemplified by the greatly increased size of transgenic mice with elevated levels of growth hormone in their serum[2-4]. Transgenic mice usually transmit the transgenes to their offspring, allowing large numbers of transgenic animals to be produced readily, by conventional breeding.

Gene transfer has considerable potential for the genetic improvement of farm animals. [5-9]. However, the generation of transgenic livestock not only presents a considerable technical challenge, but transferred genes must also confer economic advantage. While it is not yet clear which genes will have beneficial effects on conventional animal production traits (eg. reproductive performance, growth rate, carcase composition), we envisage the introduction of novel traits by gene transfer. One such trait may be the production of high-value proteins in the lactating mammary gland and their secretion into the milk for ease of collection[6-11]. The feasibility of such an approach is demonstrated by the secretion of foreign proteins into the milk of particular transgenic mice[11,12].

In view of the high cost and protracted time scale of generating transgenic cattle, we elected to pursue experiments in sheep[9,10]. While there have been few reports of transgenic livestock to date, two laboratories have generated transgenic pigs with efficiencies approaching those obtained in mice[9,13]. In contrast, two large experiments attempting gene transfer into sheep resulted in only one transgenic animal[5,7]. This sheep carried a single copy of the injected DNA, rearranged such that the gene could not be expressed[5]. These results have led to the speculation that sheep may be particularly refractory to gene transfer[7].

We report here gene transfer into sheep by microinjection of DNA into fertilized eggs, at a frequency similar to that obtained in mice. In each case the injected DNA was incorporated without detectable rearrangement, and three transgenic sheep have been shown to transmit the transgene to their progeny. Two gene constructs transferred into sheep (BLG-FIX and BLG-α1AT) are designed to direct the synthesis of human blood coagulation factor IX and human α1-antitrypsin in the lactating mammary gland and their secretion into milk.

RESULTS AND DISCUSSION

Production of transgenic sheep. Many factors influence the efficiency of production of transgenic mice by microinjection of DNA. Of particular importance are the site of injection of the DNA (pronuclei) and the form and concentration of the DNA[14] (linear, 1–2 μg/ml). We therefore chose to inject appropriate concentrations of linear DNA into the pronuclei of single-cell sheep eggs. Due to a lack of control of the precise timing of sheep early development, a number of 2- and 4-cell eggs were obtained, and these were also injected. Unlike mouse eggs, sheep eggs are semi-opaque and pronuclei and nuclei are not readily visible. Preliminary experiments showed that differential interference contrast (DIC) microscopy may be used to visualise pronuclei and nuclei in eggs that have been centrifuged, and that a proportion of such eggs are viable (data not shown). Subsequently, pronuclei were seen in untreated eggs (Fig. 1) and we have found that with careful DIC microscopy, pronuclei are visible in over 90% of eggs which have them (I. Wilmut and J. P. Simons, unpublished). A small number of eggs were injected after centrifugation, and centrifugation was discontinued when it was found that pronuclei could be visualized reliably without it. Details of the eggs injected and the animals obtained are summarized in Table 1.

Three DNA constructs (Fig. 2) were microinjected into sheep eggs. The first construct, pMK[15], contains the mouse metallothionein-1 (MT) promoter linked to the herpes simplex virus thymidine kinase (HSV-TK) gene in plasmid pBR322. This construct has been shown to function in transgenic mice[15,16]. The other constructs (BLG-FIX and BLG-α1AT) were based on the sheep gene encoding β-lactoglobulin (BLG), a major milk protein. The cloned sheep BLG gene[17] has been shown to be expressed specifically in the mammary glands of lactating

transgenic mice, directing the secretion of sheep BLG into mouse milk[11]. BLG-FIX and BLG-αlAT have cDNA sequences encoding human factor IX (FIX) or human α1-antitrypsin (αlAT) inserted into the 5' untranslated region of the BLG gene. These genes were designed to direct the synthesis of the human proteins in the sheep mammary gland and secretion into milk. pMK was linearized prior to microinjection, while the BLG-FIX and BLG-αlAT fusion genes were excised from the plasmid vectors because vector sequences have been shown to interfere with the expression of some transgenes[12-15].

A total of 511 microinjected eggs were implanted into recipient ewes and 119 fetuses were identified by ultrasound scanning at mid-gestation. DNA was obtained from 92 live-born lambs and from 17 dead fetuses (Table 1) and analysed by Southern blotting. Six transgenic lambs were identified (Fig. 3), and confirmed as such by blotting of DNA prepared from a second, independent blood sample. None of the fetuses analyzed was transgenic. One transgenic sheep carries pMK, four carry BLG-FIX and one BLG-αlAT. The estimated numbers of copies of DNA integrated in these animals ranges from 1 to 40 copies per cell (Table 2). All the transgenic sheep developed from eggs that had been injected at the one-cell stage; none of the 25 animals derived from the injection of 2- or 4-cell eggs was transgenic, suggesting that injection at these stages is less efficient for the generation of transgenic sheep. Brinster et al.[16] have found that injection into 2-cell eggs is less efficient for the generation of transgenic mice, in pigs the reverse may be true[17].

The frequency with which we obtained transgenic sheep is within the range of frequencies obtained by others with mice[14,18]. Two previous attempts to transfer DNA into sheep were less successful: 1) reimplantation of 1032 injected eggs generated 73 lambs and fetuses, one of which was transgenic[8], and 2) no transgenic fetuses were found among 71 analysed[9]. We obtained six transgenic sheep from 511 eggs that were injected and transferred (1.2% of eggs transferred). This improved frequency reflects both higher survival of injected eggs following reimplantation and a higher frequency of incorporation of the foreign DNA. It may be relevant that the protocol of Hammer et al.[8] included an extended period (>5h) of egg culture *in vitro*, whereas we reimplanted eggs as soon as possible after microinjection. It may also be significant that both of the earlier studies employed metallothionein-/growth hormone (MT/GH) fusion genes. Expression of MT/GH genes in transgenic mice is known to have deleterious pleiotropic effects on fertility[19] and it is possible that MT/GH expression may be detrimental to the ovine fetus. The fact that the single transgenic lamb obtained by Hammer et al.[8] carried a rearranged copy of the MT/GH gene is consistent with such an effect.

FIGURE 1 Visualization of sheep pronuclei. A single-cell sheep egg with both pronuclei clearly visible is shown immobilized on a holding pipette, for microinjection. The microinjection pipette is to the right. This is an exceptionally clear example: in most eggs the pronuclei are less distinct, and on occasion are invisible. In indistinct cases, pronuclei may be positively identified by visible distortion of the nuclear membrane on insertion of the microinjection pipette, and by the swelling which occurs on injection of DNA. Microscopy was performed with a Nikon Diaphot system.

Integrated transgenes are unrearranged. To assess the integrity of the incorporated transgenes, we performed Southern hybridization analyses on genomic DNA from each transgenic lamb. Transgenic animal 5LL229 was derived from an egg injected with BamHI-linearized pMK. Southern analysis of DNA from this animal after digestion with restriction endonucleases which cleave once or twice within pMK (Fig. 2), revealed hybridizing bands of sizes and intensities consistent with integration of a single unrearranged molecule of pMK (Fig. 3), which is intact at least from the left hand PvuII site to the EcoRI site near the right hand end (Fig. 2). In transgenic mice carrying pMK, the transgene is often, but not always expressed in the liver[13,16]. Despite the integrity of the pMK DNA integrated in 5LL229, no HSV-TK RNA was detected by Northern blotting of RNA from a liver biopsy (data not shown).

A similar analysis of transgene structure was performed on DNA from the four transgenic animals carrying the BLG-FIX fusion gene. The fusion gene could not be used as a hybridization probe because it contains repeated sheep DNA sequences within the BLG fragment. Instead, we probed separately with human FIX and sheep BLG cDNA sequences. The construct contains several EcoRI and BamHI sites (Fig. 2b). Cleavage with these enzymes gave the predicted hybridizing bands in all cases (Fig. 3b). In three animals, 6LL225, 6LL231 and 6LL240, the hybridization intensities revealed the presence of multiple copies of the injected DNA segment. Cleavage with Hin-

TABLE 1 Summary of eggs injected and animals obtained.

Construct	Stages of Injected Eggs					Animals Resulting	
	1-Cell	2-Cell (a)	2-Cell (b)	2-Cell (c)	4-Cell (d)	Lambs Born	Fetuses
pMK	93	6	14	22		26	9
pMK (e)	15			3		3	
BLG-FIX	252	27	17	4	7	52	5
BLG-αlAT	42	2			5	11	3
Total	402	35	31	31	12	92	17
Grand Total			511			109	

a: both cells injected, both cells survived.
b: both cells injected, one cell survived.
c: one cell injected.
d: one, two or three cells injected.
e: eggs centrifuged before injection.

dIII, which has a single recognition site in the construct (Fig. 2b), shows that the transgene is present in each of these animals both in head-to-tail and head-to-head repeats (Fig. 2b). The DNA incorporated in these sheep appears not to have undergone rearrangement. The hybridization intensity obtained with DNA from animal 6LL239 indicates the presence of one copy of the transgene per cell; the absence of HindIII bands corresponding to head-to-head or head-to-tail tandem arrays suggests that the esumated low copy number is not due to mosaicism.

Animal 6LL273 was produced by microinjecuon of the BLG-α1AT fusion construct. The insert was incompletely excised from the plasmid vector and the DNA injected contained approximately equal amounts of the BLG-α1AT (SalI-XbaI) insert of pSS1tgXS-α1AT and XbaI-linearized plasmid. Southern analysis of HindIII-cleaved 6LL273 genomic DNA revealed multiple copies of the intact linearized plasmid organized in a tandem head-to-tail array; bands that would have arisen from integrated multiple copies of the SalI-XbaI insert without vector were not observed (Fig. 3c). Hybridizing bands observed (Fig. 3c) using enzymes cleaving within the construct (EcoRI and BamHI) were as predicted from the restriction map (Fig. 2c). No rearrangement of the integrated construct was evident (Fig. 3c).

Transgenic sheep transmit the new genes to their progeny. To date, four of the transgenic sheep described above have produced offspring, which were analysed for the presence of the transgenes. Due to their immaturity, 6LL273 was not bred and 6LL239 did not sire any lambs.

Eighteen transgenic lambs were sired by 6LL225, from a total of thirty-eight offspring. Transgenic offspring of 6LL225 fall into three classes. The majority (15/38) are similar to 6LL225, with about 40 copies of the transgene per cell, two sheep have a low copy number, and one has an intermediate copy number (Fig. 4). Two possible explanations for this unexpected pattern of inheritance are that the integrated DNA may be unstable, or that 6LL225 carries transgenes integrated into more than one chromosomal site. While multiple sites of integration have commonly been found in transgenic mice[25], the lower copy number-transgenic offspring of 6LL225 were obtained at frequencies significantly less than 50% of scorable animals (1 out of 25, and 2 out of 22; $p<0.001$ and $p<0.001$). These frequencies could be explained by mosaicism of 6LL225 for the lower copy number-integration sites, or by integration into multiple sites on a single chromosome, with recombination occasionally occurring to separate sites of integration. Both male and female transgenic offspring of 6LL225 were obtained.

Transgenic ewe 5LL229 gave birth to one non-transgenic lamb, and ewes 6LL251 and 6LL240 each had one lamb (female and male, respectively), both of which are transgenic. Ewes 6LL231 and 6LL240 both carry the BLG-FIX transgene, designed to direct synthesis of human factor IX in the mammary gland during lactation. Preliminary analysis of milk from these animals indicates the presence of human factor IX (data not shown). The patterns of hybridization obtained with DNA from the offspring of 6LL231 and 6LL240 are indistinguishable from those of their mothers, suggesting that the integrated transgenes were inherited intact.

Some 30% of transgenic mice obtained from microinjected single-cell eggs have mosaic germlines and transmit the transgene to progeny at low frequency[26]. The remainder transmit the DNA at high frequency. To realize the full potential of gene transfer in livestock, it is essential that the integrated transgene is transmitted to the progeny of transgenic founder animals. Transgenic animals

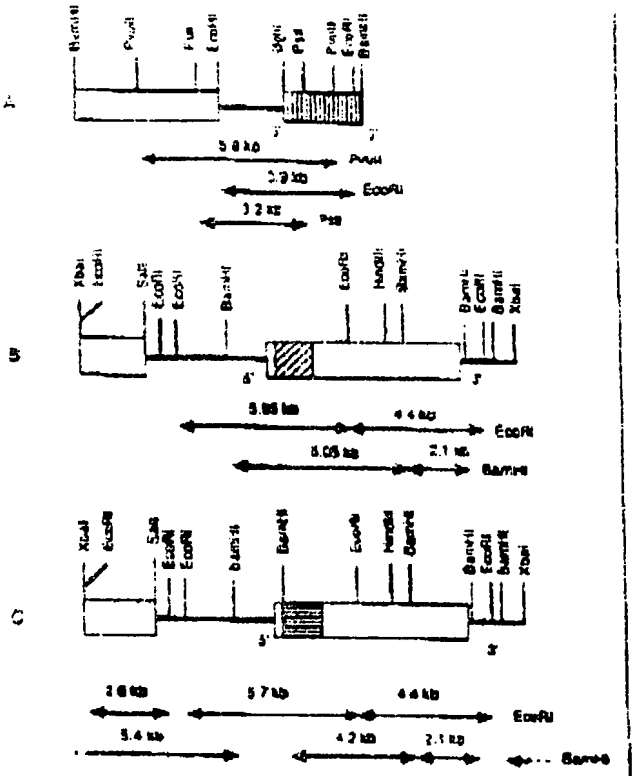

FIGURE 2 Restriction maps of microinjected constructs. A. Plasmid pMK[19]. Stippled box: pBR322 vector; solid line: mouse MT-1 promoter segment; vertical shaded box: HSV TK gene. Fragments relevant to the analysis of the transgenic sheep are indicated below the map. pMK was injected after linearization with BamHI. B. BLG-FIX. BLG-FIX, the insert of pSS1tgXS-FIX, is a 10.5 kb XbaI-SalI fragment from BLG genomic clone SS1[11,17], comprising the 4.9 kb transcripuon unit and flanking sequences, with a cDNA encoding human Factor IX (derived from pS'G3'cVI[27], 1.55 kb NheI-HindIII fragment) inserted into a PvuII site in the 5' untranslated region of the BLG gene. The predicted transcript of this fusion gene is a bicistronic mRNA (5'-FIX-BLG-3'). Stippled box: vector pPoly1[28]; open boxes: BLG transcripuon unit; solid lines: BLG flanking sequences; diagonal shaded box: FIX cDNA sequences. Beneath the map, the origins of the BamHI and EcoRI fragments detected (Fig. 3b) with the FIX and BLG cDNA probes are shown. The fragment injected was the Xba-SalI insert. C. BLG-α1AT. BLG-α1AT, the insert of pSS1tgXS-α1AT is analogous to BLG-FIX except that in place of the FIX cDNA a human α1AT cDNA was inserted (p5α1ppg (a gift of R. Cortese), 1.5 kb TaqI-BstNI fragment). Horizontal shaded box: α1AT cDNA sequences. The fragment was injected as a mixture of XbaI-SalI insert and XbaI-linearized pSS1tgXS-α1AT. Beneath the map, the origins of the BamHI and EcoRI fragments detected (Fig. 3c) by probing with α1AT and BLG cDNA clones are shown.

obtained by injecting 2- or 4-cell eggs are most likely to be mosaic. This is undesirable, especially in species which have small litters such as sheep and cows. Together with our failure to obtain transgenic sheep by injection of 2- and 4-cell eggs, this argues for injecuon only at the pronuclear stage.

In conclusion, we have demonstrated that gene transfer into the germ-lines of sheep may be reliably accomplished by microinjection of DNA into pronuclei of fertilized eggs. Transgenes are integrated without rearrangements, and the efficiency with which transgenic sheep may be obtained is sufficiently high to suggest that gene transfer into this species is practicable.

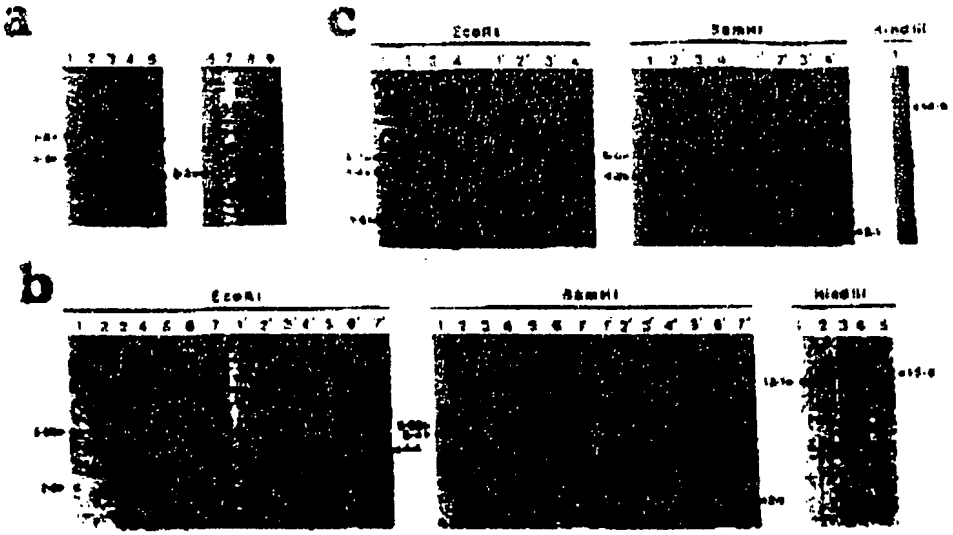

FIGURE 3 Southern analysis of transgenic sheep DNA. A. pMK. lane 1, control sheep DNA; lanes 2, 4, 6, and 8, control DNA plus 1 copy equivalent of pMK; lanes 3, 5, 7 and 9, DNA from 6LL229. The DNA was digested with EcoRI (lanes 1, 4, and 5), PvuII (lanes 2 and 3), PstI (lanes 6 and 7) and BglII (lanes 8 and 9). The hybridization probe was plasmid pTK1[39]. PvuII, EcoRI and PstI each cleave pMK twice and digests of 6LL229 DNA yielded the predicted hybridizing internal fragments: PvuII, 5.8 kb; EcoRI, 3.9 kb; PstI, 3.2 kb (see Fig. 2a). The BglII digest gave two hybridizing fragments neither of which coincides with linear pMK. These are presumed to originate from fragments extending from the single internal BglII site in pMK to flanking chromosomal BglII sites. B. BLG-FIX. DNA samples were cleaved as indicated. EcoRI- and BamHI-cleaved DNA: lanes 1, control sheep DNA; lanes 2 and 3, control DNA plus 1 and 5 copy-equivalent of pSSIigXS-FIX; lanes 4–7: DNA from transgenic sheep 6LL225, 6LL231, 6LL239 and 6LL240. The filters were hybridized with the human FIX cDNA clone p5'G3'cVI (lanes 1–7) and subsequently with the BLG cDNA clone pβ-Lg931[36] after stripping (lanes 1'–7'). Each transgenic sheep yields FIX-hybridizing bands of 5.95 kb (EcoRI) and 6.05 kb (BamHI) identical in size with those derived from BLG-FIX, showing that the 5' ends of the integrated BLG-FIX transgenes are intact. Significant hybridization with the sheep FIX gene was not observed. Hybridization with the BLG probe revealed the predicted 4.4 kb EcoRI and 2.1 kb BamHI bands. Although the endogenous BLG genes contribute to the hybridization in these bands, the increased intensity in samples from 6LL225, 6LL231 and 6LL240 indicates that these bands derive primarily from the foreign DNA, confirming the integrity of the 3' ends of the transgenes. HindIII cleaved DNA: lane 1, 12.1 kb SalI-XbaI BLG-FIX fragment; lanes 2–5 DNA from 6LL225, 6LL231, 6LL239 and 6LL240. The probe was the FIX cDNA clone. The hybridizing 12.1 kb HindIII fragment (6LL225, 6LL231 and 6LL240), identical in size with the injected fragment, indicates a head-to-tail arrangement; the 15.6 kb fragment common to the same sheep indicates that head-to-head repeats are also present. C. BLG-α1AT. DNA samples were digested as indicated. EcoRI- and BamHI-cleaved DNA: lanes 1, control sheep DNA; lanes 2 and 3, control DNA plus 5 and 1 copy equivalents of pSSIigXS-α1AT; lanes 4, 6LL273 DNA. The filters were hybridized with human α1AT cDNA clone pδα1ppg (lanes 1–4) and, after stripping, with BLG cDNA clone pβ-Lg931 (lanes 1'–4'). 6LL273 DNA digested with EcoRI or BamHI gives α1AT hybridizing bands of 5.7 and 2.6 kb and 5.4 and 4.2 kb respectively, as obtained on cleavage of pSSIigXS-α1AT, showing the integrity of the 5' end of the fusion gene. As before, the intensity of hybridization of the 4.4 kb EcoRI and 2.1 kb BamHI fragments with pβ-Lg931 indicates their origin is primarily from the unrearranged 3' end of the transgene. HindIII cleaved 6LL273 DNA (lane 1) yields a 14.0 kb fragment hybridizing with pδα1ppg, indicating a head-to-tail arrangement of linear pSSIigXS-α1AT DNA.

EXPERIMENTAL PROTOCOL

DNA for microinjection. pMK[39] was prepared for microinjection by BamHI digestion, phenol-chloroform extraction and ethanol precipitation. BLG-FIX and BLG-α1AT fusion genes were released from the plasmid vectors by digestion with SalI and XbaI, electrophoresed on 0.5% agarose gels and electroeluted onto dialysis membranes. Isolated fragments were purified on elutip D columns (Schleicher and Schüll), redissolved at about 100 μg/ml in 0.2 μm-filtered 10mM Tris.HCl, 1 mM EDTA (pH 7.5), and stored frozen. For microinjection, the DNA was diluted to 2 μg/ml (pMK) or 1.5 μg/ml (BLG-FIX and BLG-α1AT) in 0.2-μm-filtered double distilled water. Micropipette tips used to handle the DNA were rinsed thoroughly with filtered double distilled water to remove particles that could block microinjection pipettes. The DNA solution was centrifuged at 10,000 g for 30 min.; an aliquot withdrawn from below the surface was used to fill injection pipettes.

Collection of eggs. Animals used as egg donors were ewes of proven fertility of a variety of breeds: Welsh Mountain, Scottish Blackface, Greyface (Scottish Blackface × Border Leicester), and Cheviot. Rams of proven fertility were of several breeds: Finnish Landrace × Dorset Horn, Scottish Blackface and East Friesland. Donor ewes were treated with progestagen sponges (Veramix, Upjohn Ltd., Crawley, UK.) for 12–16 days to synchronize the time of estrus. Superovulation was induced by subcutaneous injection of equine FSH[39] in 2 equal doses of 1.75 or 2.15 mg, 30h before and at the time of sponge withdrawal (noon). Ewes were tested for the onset of estrus at 0800h, 1200h, 1600h and 2000h. At estrus, each ewe was served by at least two rams. Eggs were recovered[39] by flushing oviducts with prewarmed ovum culture medium (Flow Labs., Irvine, UK) during mid-ventral laparotomy.

Microinjection of DNA and reimplantation of eggs. Microinjection was performed using essentially standard techniques[36]. Eggs were manipulated in cover-slip chambers in ovum culture medium supplemented with 20% fetal calf serum. The ends of the chambers were covered with Dow Corning 200/50cs fluid.

TABLE 2 Summary of transgenic sheep.

Lamb No.	Sex	Construct	Copy no. (Approx.)
6LL229	F	pMK	1
6LL225	M	BLG-FIX	40
6LL231	F	BLG-FIX	10
6LL239	M	BLG-FIX	1
6LL240	F	BLG-FIX	10
6LL273	F	BLG-α1AT	4

All of the eggs were injected at the pronucleus stage and none were centrifuged. Copy numbers were determined by quantitative scanning densitometry of Southern blot autoradiographs.

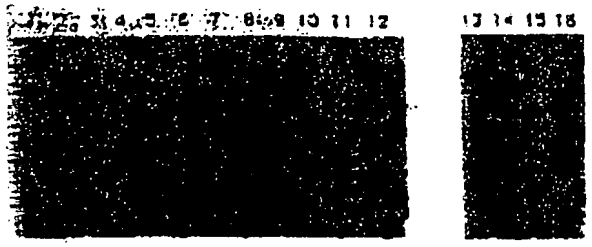

FIGURE 4 Germline transmission of incorporated DNA. Southern blot analysis of transgenes in offspring of BLG-FIX transgenic sheep 6LL225. The samples in lanes 1 to 12 were digested with EcoRI and probed with the FIX cDNA clone p5'G3'cVI. Lanes 1 to 12 contain DNA from representative progeny of transgenic sheep 6LL225, lane 12 contains DNA from 6LL225. Lanes 2, 8, 10 and 11 are representative of 20 of the 98 offspring: the transgene is not present. Lanes 1, 3, 4 and 6 are representative of 15 progeny, showing about 40 copies per cell of BLG-FIX. Lanes 5 and 9 show DNA from the two animals which have a low number of copies of the FIX sequences, and lane 7 shows DNA from the one son of 6LL225 which carries an intermediate number of copies of the transgene. In each transgenic sheep, the diagnostic 5.95 kb fragment was present. Lanes 13 to 16 contain DNA after digestion with HindIII and probing with p5'G3'cVI. The samples are from animals representative of the four classes of offspring, the same animals as shown in lanes 5 to 8. In the offspring which carry about 40 copies per cell and those which have a low copy number, the fragments diagnostic of head-to-tail (12.1kb) and head-to-head (15.0kb) repeats are both present, as found with the parent animal. Lane 15 shows DNA from the one offspring which carries about 5 copies per cell of BLG-FIX; the fragment diagnostic of head-to-head repeats is absent. Lane 16 contains DNA from a non-transgenic animal.

Microinjection pipettes were very fine (<0.5 μm) and tapered at a small angle (≤5 degrees), to minimise lethal damage to the eggs. They were pulled from thin wall borosilicate glass (1.0 mm O.D., Kwik-fill, Clark Electromedical Instruments, Pangbourne, UK). Single-cell eggs had DNA injected into one pronucleus, 2-cell eggs into both nuclei (season 1985/86) or into one nucleus (1984/85, or when only one was visualized, 1985/86). Injection of 4-cell eggs was into one, two or three nuclei (1985/86). When 2- or 4-cell eggs were injected more than once, injections were performed without repositioning the eggs on the holding pipette to avoid confusion over which cells remained to be injected. Successful injection was indicated by marked swelling of pronuclei or nuclei. Injected eggs were incubated for at least 30 min. before transfer, to allow damage to become apparent. Some eggs were centrifuged prior to injection at 10,000 g for 10 min. or for 5 min. after 30 min. incubation in medium containing 0.1 μg/ml colcemid. Egg recipients (Welsh Mountain ewes) were treated with progestagen sponges (Veramix, Upjohn, Crawley, UK) for 12–16 days to synchronise their estrus cycles with those of the egg donors. Injected eggs were implanted as deeply as possible into the oviducts of recipients which had ovulated. 1–3 eggs were transferred per recipient (1985/86 or 1–4, 1984/85 or when the recipients were one day out of synchrony 1985/86). The embryos were distributed between the oviducts.

DNA Analysis. Samples of DNA (10 μg) from peripheral blood lymphocytes were analysed by restriction enzyme digestion, gel electrophoresis (1% agarose), Southern transfer to nylon membranes (Hybond N, Amersham) and hybridization using standard procedures.

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References

1. Palmiter, R. D. and Brinster, R. L. 1986. Germline transformation of mice. Ann. Rev. Genet. 20:465–499.
2. Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C., and Evans, R. M. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611–615.
3. Palmiter, R. D., Norstedt, G., Gelinas, R. E., Hammer, R. E., and Brinster, R. L. 1983. Metallothionein-human GH fusion genes stimulate growth of mice. Science 222:809–814.
4. Morello, D., Moore, G., Salmon, A. M., Yaniv, M., and Babinet, C. 1986. Studies on the expression of an H-2K/human growth hormone fusion gene in giant transgenic mice. EMBO J. 5:1877–1883.
5. Hammer, R. E., Pursel, V. G., Rexroad, C. E., Jr., Wall, R. J., Bolt, D. J., Rhen, K. M., Palmiter, R. D., and Brinster, R. L. 1985. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683.
6. Evans, J. W. and Hollaender, A. 1986. Genetic Engineering of Animals. Plenum, New York.
7. Ward, K. A., Franklin, I. K., Murray, J. D., Nancarrow, C. D., Raphael, B. A., Rigby, N. W., Byrne, C. R., Wilson, B. W., and Hunt, C. L. 1986. The direct transfer of DNA by embryo microinjection. *In:* Proceedings 3rd World Congress Applied to Livestock Production, p. 6–11. G. E. Dickerson & R. K. Johnson, (eds.). University of Nebraska, Lincoln, Nebraska.
8. Simons, J. P. and Land, R. B. 1987. Transgenic Livestock. J. Reprod. Fertil. Suppl. 34:257–250.
9. Clark, A. J., Simons, P., Wilmut, I., and Lathe, R. 1987. Pharmaceuticals from transgenic livestock. Trends Biotechnol. 5:20–24.
10. Lathe, R., Clark, A. J., Archibald, A. L., Bishop, J. O., Simons, P., and Wilmut, I. 1986. Novel products from livestock. *In:* Exploiting new technologies in animal breeding: genetic developments. Smith, C., King, J. W. B., and McKay, J. C. (eds.). Oxford University Press, Oxford.
11. Simons, J. P., McClenaghan, M., and Clark, A. J. 1987. Alteration of the quality of milk by expression of sheep β-lactoglobulin in transgenic mice. Nature 328:530–532.
12. Gordon, K., Lee, E., Vitale, J. A., Smith, A. E., Westphal, H., and Hennighausen, L. 1987. Production of human tissue plasminogen activator in transgenic mouse milk. Bio Technology 5:1183–1187.
13. Brem, G., Brenig, B., Goodman, H. M., Selden, R. C., Graf, F., Kruff, B., Springman, K., Hondele, J., Meyer, J., Winnacker, E.-L., and Kreuzaler, H. 1985. Zuchthygiene 20:251–252.
14. Brinster, R. L., Chen, H. Y., Trumbauer, M. E., Yagle, M. K., and Palmiter, R. D. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Nat. Acad. Sci. U.S.A. 82:4438–4442.
15. Brinster, R. L., Chen, H. Y., and Trumbauer, M. 1981. Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell 27:223–231.
16. Palmiter, R. D., Chen, H. Y., and Brinster, R. L. 1982. Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. Cell 29:701–710.
17. Ali, S. and Clark, A. J. 1988. Characterization of the gene encoding β-lactoglobulin: similarity to the genes for retinol binding protein and other secretory proteins. J. Molec. Biol. In press.
18. Chada, K., Magram, J., Raphael, K., Radice, G., Lacy, E., and Costantini, F. 1985. Specific expression of a foreign β-globin gene in erythroid cells of transgenic mice. Nature 314:377–380.
19. Townes, T. M., Lingrel, J. B., Chen, H. Y., Brinster, R. L., and Palmiter, R. D. 1985. Erythroid-specific expression of human β-globin genes in transgenic mice. EMBO J. 4:1715–1723.
20. Hanover, R. E., Krumlauf, R., Camper, S. A., Brinster, R. L., and Tilghman, S. M. 1987. Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. Science 236:53–58.
21. Pursel, V. G., Miller, K. F., Pinkert, C. A., Palmiter, R. D., and Brinster, R. L. 1987. Development of 1-cell and 2-cell pig ova after microinjection of genes. J. Anim. Sci. 65(Suppl. 1):407(Abstr.).
22. Hammer, R. E., Palmiter, R. D., and Brinster, R. L. 1986. Partial correction of murine hereditary growth disorder by germ-line incorporation of a new gene. Nature 311:65–67.
23. Wilkie, T. M., Brinster, R. L., and Palmiter, R. D. 1986. Germline and somatic mosaicism in transgenic mice. Devel. Biol. 118:9–18.
24. Smith-Hatcher, A., Mills, J. S., Welch, R., and Thomas, M. 1968. Fractionation of pituitary hormones from horse pituitary glands. J. Reprod. Fertil. 17:291–303.
25. Hunter, C. L., Adams, C. E., and Rowson, L. E. A. 1955. Interbreed ovum transfer in sheep. J. Agric. Sci. 45:143–149.
26. Hogan, B., Constantini, F., and Lacy, E. 1986. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. Anson, D., Austen, D. E. G., and Brownlee, G. G. 1985. Expression of active human clotting factor IX from recombinant DNA clones in mammalian cells. Nature 315:683–685.
28. Lathe, R., Vilotte, J.-L., and Clark, A. J. 1987. Plasmid and bacteriophage vectors for excision of intact inserts. Gene 57:193–201.
29. Spandidos, D. A., and Paul, J. 1982. Transfer of human globin genes to erythroleukemic mouse cells. EMBO J. 1:15–20.
30. Campbell, P., Hue-Delahaie, D., Mercier, J.-C., Soulier, S., Vilotte, J.-L., and Furet, J.-P. 1986. Ovine β-lactoglobulin messenger RNA nucleotide sequence and mRNA levels during functional differentiation of the mammary gland. Biochimie 68:1097–1107.